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SGK1-Dependent Upregulation of Connective Tissue Growth Factor by Angiotensin II

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Key Words
Fibrosis · Serum- and glucocorticoid-inducible kinase · Human kidney fibroblasts · Connective tissue growth factor

Abstract
Angiotensin II has previously been shown to trigger fibrosis, an effect involving connective tissue growth factor (CTGF). The signaling pathways linking angiotensin II to CTGF formation are, however, incompletely understood. A gene highly expressed in fibrosing tissue is the serum- and glucocorticoid-inducible kinase SGK1. The present study explored whether SGK1 is transcriptionally regulated by angiotensin II and participates in the angiotensin II-dependent regulation of CTGF expression. To this end, experiments have been performed in human kidney fibroblasts and mouse lung fibroblasts from gene-targeted mice lacking SGK1 ($sgk1^{-/-}$) and their wild-type littermates ($sgk1^{+/+}$). In human renal fibroblasts, SGK1 and CTGF protein expression were enhanced by angiotensin II (10 nM) within 4 h. In $sgk1^{+/+}$ mouse fibroblasts, SGK1 transcript levels were significantly increased after 4 h of angiotensin II treatment. Angiotensin II stimulated both transcript and protein abundance of CTGF in fibroblasts from $sgk1^{+/+}$ mice, effects significantly blunted in fibroblasts of $sgk1^{-/-}$ mice. In conclusion, angiotensin II stimulates the expression of SGK1, which is in turn required for the stimulating effect of angiotensin II on the expression of CTGF. Thus, SGK1 presumably contributes to the profibrotic effect of angiotensin II.

Introduction

The pleotropic actions of angiotensin II include the stimulation of matrix protein formation and deposition leading to fibrosis in a variety of tissues including heart [1, 2], vascular tissue [3–6], kidney [7–13], liver [14, 15], lung [16] and retina [17]. The effect of angiotensin II is mediated by upregulation of connective tissue growth factor (CTGF) [15, 18–27]. Moreover, angiotensin II and CTGF have been shown to mediate the mitogenic effect of advanced glycation end products [28].

A candidate kinase linking the angiotensin receptors with CTGF expression is the serum- and glucocorticoid-inducible kinase SGK1 [29], a kinase originally cloned as

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a glucocorticoid-inducible gene [30] and subsequently shown to be strongly upregulated by a variety of triggers [31, 32] including mineralocorticoids [33, 34]. SGK1 is expressed in a variety of fibrosing tissues such as those affected by diabetic nephropathy [35–37], glomerulonephritis [38], cardiac fibrosis [39], lung fibrosis [40], liver cirrhosis [41], Crohn's disease [42], and fibrosing pancreatitis [43].

The present study aimed to elucidate the possible participation of SGK1 in the stimulation of CTGF expression by angiotensin II.

Materials and Methods

Preparation of Fibroblasts

Human fibroblast cell lines derived from normal kidney (TK 173) were cultured as described previously [44, 45]. Briefly, the cells were cultivated in culture flasks at 37°C in 5% CO_2 atmosphere in DMEM, containing 5.6 mM glucose supplemented with 10% fetal calf serum, 20 mM L-glutamine and 100 U/ml penicillin/100 mg/ml streptomycin and passaged every week.

To determine the role of SGK1 in angiotensin II-induced CTGF expression, fibroblasts were collected from SGK1 knockout mice (*sgk1*$^{-/-}$) and their wild-type littermates (*sgk1*$^{+/+}$) [46]. Several fibroblast preparations have been used. The yield required for Western blotting was achieved with lung fibroblasts. To harvest primary lung fibroblasts from sgk1$^{-/-}$ and sgk1$^{+/+}$ mice, whole lungs were removed under anesthesia from 8- to 14-week-old animals and transferred to 90-mm cell culture dishes containing 2 ml of DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (Gibco-Invitrogen, Karlsruhe, Germany). The tissue was cut into small pieces and cultured under standard cell culture conditions (37°C, 5% CO_2). Cell growth was observed 2–4 days after initial plating. Fibroblasts were identified by positive staining for fibronectin and used in experiments between passages 2 and 6. Animal experiments were conducted according to the guidelines of the German Animal Welfare Law and were approved by local authorities.

Western Blotting

For Western blotting, human renal fibroblasts or *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mouse lung fibroblasts were grown in 60-mm culture dishes for 32 h. Serum was removed for 18 h and cells were thereafter treated with angiotensin II, 10 nM, for 4 h. After treatment, cells were lysed and whole cell lysates (50 μg) were subjected to 10% SDS-PAGE in 10% Tris-glycine buffer. The proteins were transferred to nitrocellulose membranes and the membrane incubated for 1 h in PBS containing 5% fat-free milk and 0.1% Tween (blocking buffer) to block unspecific binding sites. The membranes were incubated overnight at 4°C with a goat polyclonal CTGF primary antibody (from Santa Cruz, Heidelberg, Germany, diluted 1:400 in blocking buffer, or from R&D Systems, Wiesbaden, Germany, at a concentration of 0.1 μg/ml) or with a rabbit polyclonal SGK1 primary antibody (from Upstate Biotechnology, Dundee, UK, diluted 1:1,000, or from Pineda, Berlin, Germany, 1:100). The CTGF antibody from R&D Systems recognizes two isoforms of about 36 and 40 kDa. The 36-kDa band was chosen for analysis. After incubation with HRP-conjugated anti-goat or anti-rabbit secondary antibodies (Santa Cruz) for CTGF and SGK1, respectively, proteins binding the antibodies were visualized with enhanced chemiluminescence according to the manufacture's instructions (Amersham, Freiburg, Germany). Membranes were probed with GAPDH antibody (Santa Cruz) to control loaded and transferred amounts of protein. Densitometric analysis of CTGF protein bands was performed using Scion Image (Scion, Frederick, Md., USA). The specific bands were referred to the staining of GAPDH or of non-specific bands. Due to differences of the exposure times of different Western blots, the densities of the bands were normalized to the values of the respective non-treated controls or wild-types.

Quantification of mRNA by Real-Time RT/PCR

For real-time PCR total RNA was isolated from cultured fibroblasts of *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice using the Qiagen RNeasy Fibrous Tissue Midi Kit (Qiagen, Hilden, Germany). SGK1 or CTGF mRNA were transcribed using Taq polymerase (Roche Diagnostics GmbH, Mannheim, Germany) and quantified by PCR using a light cycler system (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). For the detection of mouse CTGF mRNA the specific primers used were: sense: 5′-ACG ACG CCA AGG ACC GCA-3′; antisense: 5′-TTG TAA TGG CAG GCA CAG-3′. For detection of SGK1 transcript levels the specific primers used were: sense: 5′-TGTCTTGGGGCTGTCCTGTATG-3′; antisense: 5′-GCTTCTGCTGCTTCCTTCACAC-3′. The transcript levels of the housekeeping gene GAPDH were also determined for each sample using a commercial primer kit (Search LC, Heidelberg, Germany). Amplification of the target DNA was performed during 35 cycles, each 10 s at 95°C, 10 s at 68°C and 16 s at 72°C. Melting curve analysis confirmed the specificity of amplified products, which were then separated on 1.5% agarose gels to verify the size of the PCR product. Results were calculated as a ratio of the target vs. housekeeping gene transcripts.

Statistical Analysis

Data are provided as means ± SEM, n represents the number of independent experiments. All data were tested for significance using ANOVA or t-test, as appropriate, and only results with $p < 0.05$ were considered statistically significant.

Results

SGK1 and CTGF Expression Is Stimulated by Angiotensin II in a Fibroblast Cell Line

To explore whether the serum- and glucocorticoid-inducible kinase SGK1 is a transcriptional target of angiotensin II in human tissues, experiments were performed with fibroblast cell lines derived from normal kidneys. As illustrated in figure 1, after exposure to angiotensin II (10 nM) for 4 h the SGK1 protein abundance was significantly increased in the fibroblasts. Next, the expression of CTGF was analyzed (fig. 2). Incubation of the cells with

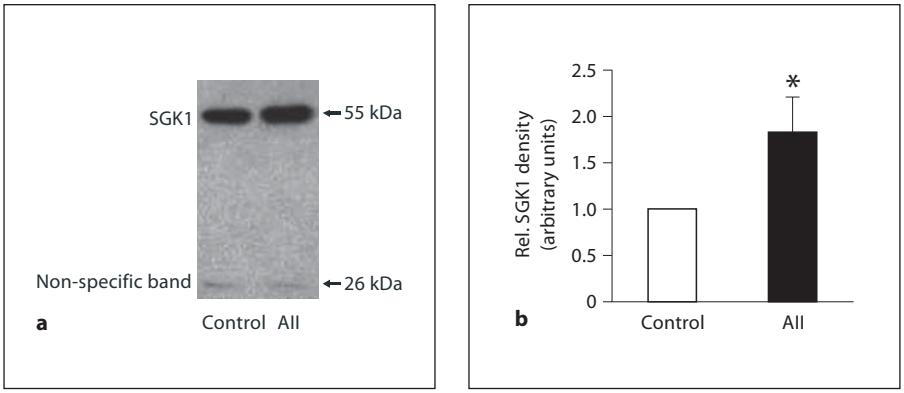

Fig. 1. Angiotensin II (AII) stimulates SGK1 expression in human fibroblasts. **a** Representative Western blot demonstrating SGK1 protein abundance after a 4-hour incubation in the absence or presence of 10 nM AII. A non-specific band served as loading control. **b** Arithmetic means ± SEM (n = 6) of SGK1 over GAPDH protein abundance or normalized to a non-specific band in fibroblasts from normal human kidneys after a 4-hour incubation in the absence (left bar) or presence (right bar) of 10 nM AII. * Significant ($p < 0.05$) difference from control.

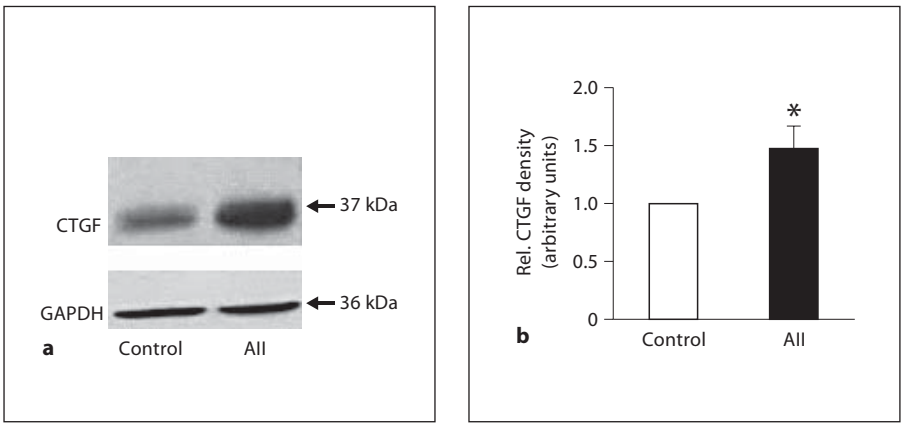

Fig. 2. Angiotensin II (AII) increases CTGF protein expression in human fibroblasts. **a** Representative Western blot demonstrating CTGF and GAPDH protein levels in human renal fibroblasts after a 4-hour treatment with 10 nM AII. **b** Arithmetic means ± SEM (n = 6–7) of CTGF over GAPDH protein abundance or normalized to a non-specific band in fibroblasts from normal human kidney fibroblasts after a 4-hour incubation in the absence (left bar) and presence (right bar) of 10 nM AII. * Significant ($p < 0.05$) difference between AII and control.

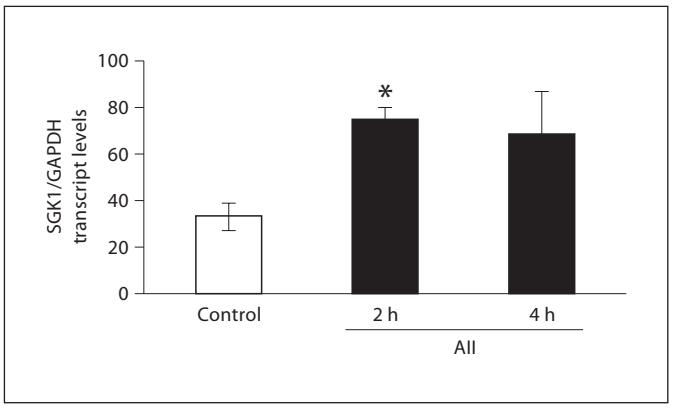

Fig. 3. Angiotensin II (AII) increases SGK1 transcript levels in lung fibroblast from wild-type mice. Arithmetic means ± SEM (n = 3) of SGK1 transcript levels in relation to GAPDH transcript levels of the same preparations of primary mouse lung fibroblasts prior to and after 2- and 4-hour treatment with 10 nM AII. * Significant difference from control.

angiotensin II for 4 h significantly increased the expression of CTGF. These observations indicate that angiotensin II upregulates the expression of SGK1 and CTGF.

Angiotensin II Increased the Expression of SGK1 in Primary Mouse Fibroblasts

To test whether SGK1 is functionally relevant for the stimulation of CTGF expression by angiotensin II, experiments have been performed in lung fibroblasts from SGK1 knockout mice (*sgk1*$^{-/-}$) and their wild-type littermates (*sgk1*$^{+/+}$). First, the effect of angiotensin II on SGK1 expression was analyzed in lung fibroblasts from wild-type mice. After exposure of primary lung fibroblasts to angiotensin II (10 nM), SGK1 mRNA was significantly increased within a 2-hour treatment (fig. 3).

CTGF Expression Is Not Stimulated by Angiotensin II in the Absence of SGK1

CTGF transcript levels were then analyzed in primary lung fibroblasts from both wild-type and sgk1$^{-/-}$ mice. As

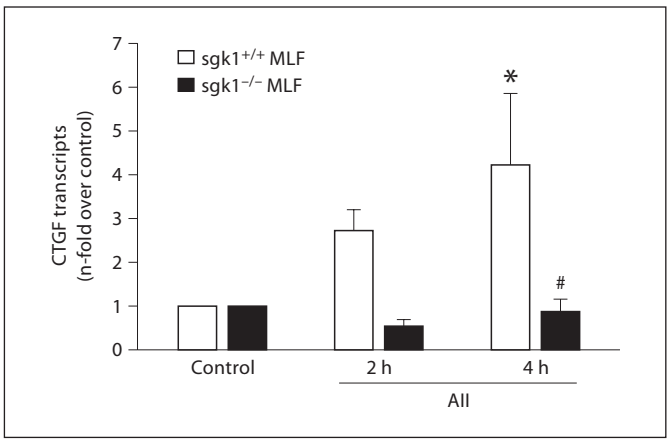

Fig. 4. Angiotensin II (AII) increases CTGF transcript levels in lung fibroblast from *sgk1*$^{+/+}$ but not from *sgk1*$^{-/-}$ mice. Arithmetic means ± SEM (n = 3) of CTGF transcript levels over GAPDH levels in primary mouse lung fibroblasts from mice lacking SGK1 (*sgk1*$^{-/-}$, closed bars) and their wild-type littermates (*sgk1*$^{+/+}$, open bars) prior to (control) and after 2- and 4-hour treatment with 10 nM AII. * Significant difference from control. # Significant difference from *sgk1*$^{+/+}$ at the same condition.

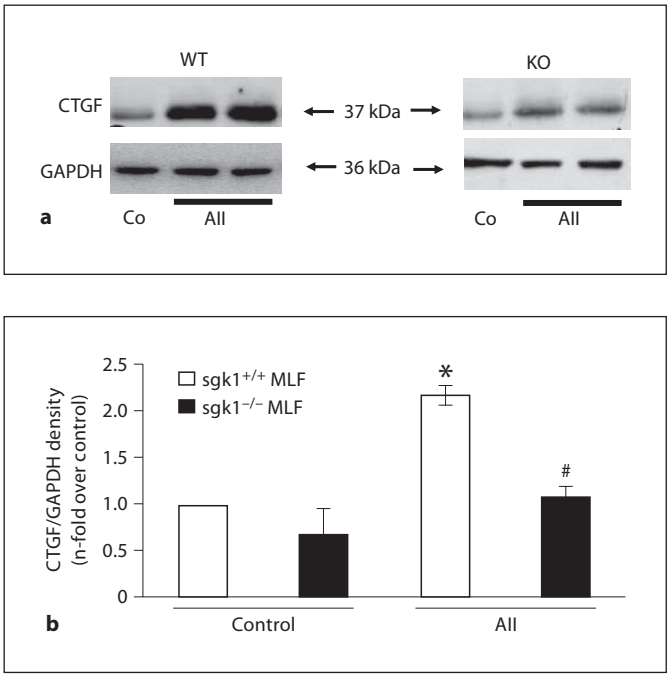

Fig. 5. Angiotensin II (AII) increases CTGF protein expression in fibroblasts from *sgk1*$^{+/+}$ but not from *sgk1*$^{-/-}$ mice. **a** Representative Western blots demonstrating CTGF and GAPDH protein levels in primary mouse lung fibroblasts from mice lacking SGK1 (sgk1$^{-/-}$, KO, right panel) and their wild-type littermates (sgk1$^{+/+}$, WT, left panel) prior to (Co) and after 4-hour treatment with 10 nM AII. **b** Arithmetic means ± SEM (n = 4) of CTGF over GAPDH protein abundance in fibroblasts from sgk1$^{-/-}$ (closed bars) and sgk1$^{+/+}$ (open bars) mice prior to (control) and after 4-hour treatment with 10 nM AII. * Significant difference from the respective untreated cells. # Significant difference between sgk1$^{+/+}$ and sgk1$^{-/-}$ mice in the presence of AII.

illustrated in figure 4, basal CTGF transcript levels were not different between *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ fibroblasts. Angiotensin II, 10 nM, however, increased CTGF transcript levels in wild-type but not in *sgk1*$^{-/-}$ mice fibroblasts. These observations indicate that transcriptional upregulation of CTGF requires the participation of SGK1.

When CTGF protein was quantified by Western blotting the upregulation in wild-type cells by angiotensin II was indeed mirrored by an increased amount of CTGF protein in these cells. In *sgk1*$^{-/-}$ mouse fibroblasts, in contrast, CTGF protein levels were not significantly increased by angiotensin II (fig. 5). These results strongly indicate that the stimulation of CTGF protein expression by angiotensin II depends on the presence of SGK1.

Discussion

The present observations demonstrate that the serum- and glucocorticoid-inducible kinase SGK1 is a transcriptional target of angiotensin II. Thus, angiotensin II upregulates SGK1 expression not only by increasing aldosterone release and subsequent mineralocorticoid stimulation of SGK1 transcription [33, 34], but as well by a more direct stimulation of SGK1 transcription.

The present observations further reveal the participation of SGK1 in the signaling mediating the stimulating effect of angiotensin II on the transcription and protein expression of CTGF [15, 18–27]. CTGF, a member of the CCN (ctgf/cyr61/nov) gene family [47], is a key mediator of matrix protein formation [48, 49]. Loss of function mutations of CTGF are lethal partly due to major skeletal defects as a result of impaired matrix remodeling [50]. CTGF is upregulated in several fibrotic diseases such as scleroderma [51], cardiac fibrosis [26, 27], hepatic fibrosis [52] and diabetic nephropathy [53]. CTGF has been demonstrated to upregulate several profibrotic factors such as collagen, integrin α5 and fibronectin [54]. SGK1-dependent upregulation of CTGF may participate in those fibrosing diseases where excessive SGK1 transcription has

been observed, such as diabetic nephropathy [35–37], glomerulonephritis [38], cardiac fibrosis [39], lung fibrosis [40], liver cirrhosis [41], Crohn's disease [42] and fibrosing pancreatitis [43].

To the extent that SGK1 is upregulated by mineralocorticoids, it could similarly participate in the stimulation of fibrosis by mineralocorticoid excess, which, for instance, has been shown to induce cardiac fibrosis [55, 56] in a pressure-independent manner via cardiac mineralocorticoid receptors [57, 58]. Inhibition of those receptors would abrogate the aldosterone-induced fibrosis but favor increase of angiotensin II release and thus promote angiotensin II-induced fibrosis. SGK1 has indeed been shown to participate in the stimulation of cardiac CTGF formation during mineralocorticoid excess [39]. Moreover, SGK1 has been shown to potentiate the stimulating effect of hyperglycemia on matrix protein formation [35].

Mechanisms linking SGK1 with CTGF expression could at least in theory involve nuclear factor NFκB [31]. SGK1 associates with and activates IκB kinase β (IKKβ), which in turn phosphorylates IκBα, leading to degradation of IκBα and thus activation of NFκB [59]. The stimulating effect of the mineralocorticoid DOCA on cardiac CTGF indeed requires both, SGK1 and NFκB [39]. Angiotensin II similarly signals through NFκB [60–62], but may not require NFκB for stimulation of fibrosis [63]. SGK1 further phosphorylates glycogen synthase kinase 3β, an effect, however, apparently not critical for cardiac fibrosis [64]. Moreover, SGK1 may be effective through its well-known role in the regulation of channels and transporters [31, 65–67]. In any case, additional experimentation will be necessary to elucidate whether the SGK1-dependent pathway stimulated by angiotensin II involves more than NFκB.

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