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...
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% CO₂ atmosphere in DMEM, containing 5.6 mM glucose supplemented with 10% fetal calf serum, 20 mM l-glutamine and 100 U/ml penicillin/l00 mg/ml streptomycin and passed 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 mice 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 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′-AGG AGG CCA AGG ACC GCA-3′; antisense: 5′-TTG TAA TGG CAG GCA CAG-3′.

**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
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<sup>−/−</sup>) and their wild-type littermates (sgk1<sup>+/+</sup>). 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<sup>−/−</sup> mice. As
SGK1-Dependent Upregulation of CTGF by Angiotensin II

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.

Discussion

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

SGK1-Dependent Upregulation of CTGF by Angiotensin II

Kidney Blood Press Res 2008;31:80–86

83
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 IkB kinase β (IKKβ),

which in turn phosphorylates IkBα, leading to degradation of IkBα 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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SGK1-Dependent Upregulation of CTGF by Angiotensin II

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