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Stimulation of serum- and glucocorticoid-regulated kinase-1 gene expression by endothelin-1

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Abbreviations:
DIG, digoxigenin
ENaC, epithelial Na+ channel
ET-1, endothelin-1
PBST, phosphate-buffered saline/Tween-20
RT-PCR, reverse transcription polymerase chain reaction
SGK1, serum- and glucocorticoid-regulated kinase-1
SNX, subtotal nephrectomy
TBS, Tris-buffered saline

ABSTRACT

The serum- and glucocorticoid-regulated kinase-1 (SGK1) participates in the regulation of sodium homeostasis and blood pressure by mineralocorticoids. Aldosterone rapidly induces SGK1 transcription, which contributes to the activation of renal epithelial sodium channels. Another important regulator of blood pressure is the vasoactive hormone endothelin-1 (ET-1) that is systemically upregulated in chronic renal failure. In the present study, we investigated whether ET-1 modulates SGK1 expression, and thereby might explain some of its hypertensive effects. As assessed by real-time PCR analysis, ET-1 triggered the rapid increase of SGK1 mRNA levels in A-10 smooth muscle cells and also in intact aortas of adult rats. In A-10 cells transcriptional activation was associated with a more than 6-fold upregulation of SGK1 protein expression and in similar range as found after treatment with aldosterone. A stimulatory effect of ET-1 was not only observed in isolated cells, but also in an animal model. Upon subtotal nephrectomy (SNX) of rats, myocardial ET-1 levels strongly increased, which was followed by a more than 2-fold induction of SGK1 expression in the left ventricle. The myocardial upregulation of SGK1 was completely abrogated by a specific ET_A receptor antagonist, thereby substantiating the in vivo role of ET-1 in SGK1 expression. Thus, these data demonstrate that ET-1 increases expression of SGK1 in vivo and in vitro, and therefore indicate that SGK1 upregulation might be involved in ET-1-dependent regulation of blood pressure and cardiac modelling during mild renal failure.

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1. Introduction

Endothelin-1 (ET-1) is a major peptide member of the endothelin family and has been originally identified as a potent vasoconstrictor. ET-1 has various additional biological effects such as regulation of proliferation and function of vascular smooth muscle cells as well as other functions within and outside the cardiovascular system [1]. The biological effects of ET-1 are transduced through two types of receptors, termed ET_A and ET_B. Both receptors exert their effects through guanine nucleotide-binding proteins leading to the activation of phospholipase C, protein kinase C and other second messenger systems. Enhanced ET-1 levels increase the vascular tone and favour the development of hypertension [2–5]. This is also exemplified in chronic renal failure, which is associated with increased ET-1 concentrations leading to high blood pressure and left ventricular hypertrophy [6]. Conversely, ET receptor antagonists have proven effective in antihypertensive treatment [1,7,8].

ET-1 has been shown to interact with mineralocorticoids such as aldosterone [9,10] which is another major regulator of blood pressure, at least partially because of its capacity to control renal sodium absorption. Following binding to the mineralocorticoid receptor aldosterone stimulates sodium entry via the renal epithelial Na^+ channel ENaC, which participates in the ''fine tuning'' of Na^+ excretion and blood pressure control [11–16]. Recent evidence has demonstrated that the serum- and glucocorticoid-inducible kinase SGK1 is an important mediator of this aldosterone response [11]. SGK1 is a serine/threonine protein kinase whose mRNA is rapidly induced when cells are exposed to serum or glucocorticoids [17]. SGK1 activity is regulated at two different levels, i.e. the induction of mRNA transcription [17,19] and the subsequent activation by phosphorylation [18]. In addition to serum and corticoids, hyperosmolarity [20,21], follicle-stimulating hormone [22], various growth factors [19,23,24] as well as increased calcium concentrations [19,25] enhance transcription of the SGK1 gene. Although the mechanisms of SGK1 gene induction remained poorly understood, experimental evidence points to the involvement of cAMP [22] and p38 kinase [20]. Several intracellular target proteins that are phosphorylated by SGK1 have been identified, indicating that SGK1 is involved in the control of gene transcription, cell cycle progression and apoptosis [26,27]. In particular, SGK1 plays an important role in activating certain potassium, sodium and chloride channels [19], suggesting an involvement in the regulation of processes such as cell volume control [28], neuronal excitability [29] and renal sodium excretion [19]. Moreover, sustained high levels of SGK1 protein and activity may contribute to conditions such as hypertension [30] and diabetic nephropathy [31]. Thus, SGK1 is an appealing candidate for deranged blood pressure regulation and may contribute to the development of hypertension [30].

Since both the ET-1 and aldosterone systems are potent regulators of the blood pressure and, moreover, use at least partially overlapping signalling pathways, we investigated in the present study the effect of ET-1 on SGK1 expression in isolated smooth muscle cells and aortic rings of adult rats. Furthermore, the interaction of aldosterone and ET-1 in the regulation of SGK1 expression was examined. We further used an animal model of renal insufficiency to investigate whether enhanced ET-1 levels also affect the mRNA expression of SGK1 in myocardial tissue in vivo.

2. Materials and methods

2.1. Cell culture

A-10 cells, derived from embryonic rat thoracic aortal smooth muscle, were obtained from the 'Deutsche Sammlung für Zellkulturen' (Braunschweig, Germany) and maintained in Dulbecco's modified essential medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated newborn calf serum. A-10 cells were used, as they only express ET_A receptor subclasses and, unlike primary cells from rat aorta, are characterized by a stable phenotype in culture [32]. Cells were set on serum-free growth medium containing transferrin and thyreoglobin 24 h prior to the experiments in order to exclude effects of the calf serum. Afterwards, the medium was exchanged and the cells were stimulated with ET-1 (10^{-7} mol/L, Sigma, Deisenhofen, Germany) or aldosterone (10^{-7} mol/L, Sigma) for various time periods. In the co-incubation experiments aldosterone and ET-1 were added simultaneously to the cells and incubated for the indicated time period. The mineralocorticoid antagonist spironolactone (10^{-7} mol/L, Sigma) alone was incubated for 120 min with A-10 cells. In the experiments with the ET_A receptor antagonist LU 302146 (10^{-6} mol/L, Knoll, Ludwigshafen, Germany) or spironolactone in combination with the agonists, the antagonists were preincubated for 30 min with the cells, before the agonists were added for 2 h.

2.2. Animal experiments

Male Sprague–Dawley rats weighing approximately 200 g were purchased from Charles River (Kisslegg, Germany) and housed individually. All animals except the untreated controls underwent a two-step 5/6 subtotal nephrectomy (SNX). First, the right kidney was removed under anesthesia with ketamin (60–80 mg/kg bw) and xylazine (5–15 mg/kg bw). One week later, the lower and upper poles of the remaining kidney were dissected. After surgery, one group of animals received no treatment; the others were treated with the selective ET_A receptor antagonist LU 302146 (30 mg/kg bw/d). All medications were administered orally in the drinking water. The animals received a low-salt diet (sodium 190 mg/kg, Altromin, Lage, Germany) to prevent the development of hypertension. After 12 weeks of treatment, the rats were killed and the hearts and thoracic aortas were explanted.

2.3. Culture of aortic rings

For in vitro culture of aortic rings, aortas from adult male Sprague–Dawley rats were used. After sacrificing the rats, the aortas were carefully removed to avoid stretching and cleaned from periadventitial tissue. The aortic region from the arch to

the diaphragm was then cut into rings of 3–4 mm length, which were placed in six-well-plates. After allowing the aortic rings to dry at 37 °C in a 5% CO_2 atmosphere for 30 min, 3 mL serum-free Dulbecco's modified essential medium supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin were added to each well. The aortic rings were incubated with ET-1 (10^{-7} mol/L) 20 h later for the indicated time. The ET_A receptor antagonist LU 302146 and the ET_A/ET_B receptor antagonist LU 302872 were added 30 min prior to the incubation with ET-1.

2.4. RNA preparation and reverse transcription PCR

For reverse transcription polymerase chain reaction analysis (RT-PCR), RNA was extracted as described [33,34] using the RNAzol B kit (Wack Chemie, Wertheim, Germany). First strand cDNA was synthesized from 2 μg total RNA in 50 μL RT buffer (20 mmol/L Tris–HCl, pH 8.3, 50 mmol/L KCl, 3 mmol/L $MgCl_2$, 0.1 mol/L dithiothreitol) with 60 U RNAsin (Amersham-Pharmacia, Freiburg, Germany), 25 mmol/L dNTPs and 100 U Moloney Murine Leukemia Virus reverse transcriptase (Gibco/BRL, Eggenstein, Germany). Reverse transcription was performed by random-priming with hexamers oligo $d(T)_{16}$ (50 pmol/μL). The samples were incubated for 10 min at 25 °C, afterwards at 48 °C for 30 min, before they were heated to 95° C for 5 min and then cooled to 4 °C in a thermal cycler (DNA Engine PTC-200, MJ Research, Watertown, USA).

2.5. Real-time semiquantitative PCR

Total RNA was isolated from A-10 cells, aortic rings and rat myocardium as described using RNAzol (Tel-Test, Friendswood, USA) [32,33]. Aliquots of total RNA (2 μg/50 μL) were reverse-transcribed in a thermal cycler with TaqMan® Reverse Transcription reagents (Applied Biosystems, Foster City, USA) in a 50 μL volume containing 2.5 μmol/L oligo $d(T)_{16}$, 62.5 U Multi Scribe™ reverse transcriptase, 20 U RNase inhibitor, 5.5 mmol/L $MgCl_2$ and 2 mmol/L dNTP mixture at 25 °C for 10 min, 48 °C for 30 min and 95 °C for 5 min. Subsequently, cDNA samples (2 μL) were amplified in 20 μL of SYBR®-Green-PCR-Master Mix (Applied Biosystems) and 300 nmol/L of the respective forward and reverse primers using an ABI Prism™ 7000 sequence detection system (Applied Biosystems) and 40 cycles of the following conditions: 2 min at 50 °C, 10 min at 95 °C, 15 s at 95 °C and 1 min at 60 °C. The relative mRNA expression of the treated and untreated cells was determined by the $2^{-\Delta CT}$ method. All reactions were performed in triplicates. The following primers were designed with Primer Express® Software v2.0 and purchased from Invitrogen (Karlsruhe, Germany): SGK1 (GenBank accession no. NM_019232) forward: 5′-GAGGGAGCGCTGCTTCCT-3′, reverse: 5′-ACCCAAGGCACTGGCTATTTC-3′, pre–pro-ET-1 (GenBank accession no. NM_012548), forward: 5′-ATCAGCAGCTGGTGGAGGG-3′, reverse: 5′-GATGCTGTTGCTGATGGCCT-3′, GAPDH (GenBank accession no. NM_017008), forward: 5′-AACTCCCTCAAGATTGTCAGCAA-3′, reverse: 5′-GGCTAAGCAGTTGGTGGTGC-3′. An aliquot that served as an internal control for each experiment was fractionated on 1.2% agarose gels containing 0.01% ethidium bromide and visualized under UV light.

2.6. SGK1 cDNA cloning and in situ hybridization of smooth muscle cells

In situ hybridization was performed as previously described [33,35]. The cDNA of rat SGK1 was obtained from A-10 cells by RT-PCR using the sense primer 5′-GGAACAGATGCCAGTGTGCT-3′ and the antisense primer 5′-CCAGACAGCAAGAAGAGG-CA-3′. The 541-bp cDNA fragment of SGK1 (bp 503–1043) was cloned into the multiple cloning site of pGEM-T (Promega, Heidelberg, Germany) flanked by Sp6 and T7 promoters. The insert was used as template for the generation of sense and antisense RNA probes and labelled with digoxigenin (DIG)-conjugated UTP (Roche Molecular Biochemicals, Mannheim, Germany). Cells were fixed with 2% formaldehyde and washed in PBS. Prehybridization buffer (50% formamide, 5% dextransulfate, 500 μg/mL yeast t-RNA, 200 μg/mL hering sperm DNA, 10% DTT, 5 × SSC [0.3 mol/L NaCl, 0.3 mol/L sodium citrate], 1 × Denhardt's solution) was applied to the cells for 3 h. The buffer was replaced by hybridization buffer (5 μg/mL DIG-labelled RNA probe in prehybridization buffer) for 12 h at 46 °C. Slides were washed in 2 × SSC for 15 min at 42 °C, in 1 × SSC at 37 °C for 15 min, in 0.5 × SSC at 37 °C, in 0.5 × SSC for 30 min and then in PBS/0.25% Tween-20 (PBST). Slices were blocked with 0.5% BSA and sheep serum (1:50) in PBST for 30 min. The gold-labelled anti-DIG antibody (1:30) was applied to the slides in 0.5% BSA/PBST for 30 min. After washing in PBST a silver enhancement reagent (Roche Molecular Biochemicals) was applied for 20 min, and the cells were counterstained with eosin.

2.7. Western blot analysis

Cell lysates (10 μg protein) were mixed with an equal volume of sample buffer (100 mmol/L Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol and 0.2% bromophenol blue) and then separated under reducing conditions on 6% SDS polyacrylamide gels according to Laemmli [36]. Subsequently, proteins were transferred at 100 V for 30 min (2.5 mA/cm^2) to nitrocellulose membranes (Amersham) using an electrophoretic transfer cell (Hoefer, Freiburg, Germany). Nitrocellulose filters were blocked for 12 h in Tris-buffered saline (TBS, 10 mmol/L Tris–HCl, pH 8.0 and 150 mmol/L NaCl) containing 5% skimmed milk powder. Subsequently, the membranes were incubated at room temperature for 2 h with the primary antibody against SGK1 (Upstate, Hamburg, Germany), diluted 1:1000 with TBS containing 1% BSA (TBS/BSA). After several washing steps in TBS/BSA, peroxidase-conjugated anti-rabbit IgG was added for 1 h. Following extensive washings, the reaction was developed by enhanced chemiluminescent staining using ECL reagents (Amersham-Pharmacia). The X-ray films were densitometrically scanned and quantified using the Image Master 1.1 software (Amersham-Pharmacia).

2.8. Statistical analysis

Statistical analyses were performed using one-way ANOVA, followed by Dunnett's test to compare results in which two or more treatment groups had to be compared with the controls [32,34]. $p < 0.05$ Were considered to denote statistically

significant differences. The data are presented as the percentage of change compared to control cultures. Each experiment was performed with the indicated replicates. The mean ± S.E.M. of data was used for statistical comparison of three to eight different sets of experiments.

3. Results

3.1. Endothelin-1 increases SGK1 mRNA expression in smooth muscle cells

To investigate the effect of ET-1 on SGK1 expression, smooth muscle cells were incubated with ET-1 (10^{-7} mol/L) for various time points. As assessed by real-time RT-PCR analysis, SGK1 mRNA levels increased significantly (122 ± 4.2%, $n = 3$, $p < 0.05$) within 30 min of ET-1 incubation and reached a maximum after 120 min (217 ± 7.6%, $n = 3$, $p < 0.01$, Fig. 1A). After ET-1 incubation for 180 min mRNA expression of SGK1 gradually declined (135 ± 12.3%) and returned to control levels after 240 min (106 ± 8.3%). Addition of the ET_A receptor antagonist LU 302046 completely inhibited the increase in SGK1 mRNA expression (93.2 ± 3.7%, $n = 6$) induced by ET-1 incubation for 120 min (Fig. 1A). Induction of SGK1 mRNA was also verified by in situ hybridization that revealed an intensive staining of the SGK1 transcripts in ET-1-stimulated, but not in untreated A-10 smooth muscle cells (Fig. 1B).

For comparison, we performed similar experiments with aldosterone, a known inducer of SGK1 expression. Aldosterone increased SGK1 mRNA in smooth muscle cells after 1 h of incubation (60 min: 152.4 ± 6.4%, $n = 3$, $p < 0.01$; 90 min: 221 ± 7.4%, $n = 3$, $p < 0.01$; 180 min: 327.5 ± 15.1%, $n = 3$, $p < 0.01$). Co-incubation of aldosterone and the mineralocorticoid antagonist spironolactone prevented this increase (101 ± 10.3%, $n = 5$, p = n.s.), whereas spironolactone alone did not appreciably affect SGK1 mRNA expression (104.2 ± 5.9%, $n = 6$; Fig. 1C).

3.2. Endothelin-1 increases SGK1 mRNA expression in cultured rat aortic rings

To substantiate the relevance of our findings, we studied the effects of ET-1 on cultured aortic rings from adult rats. SGK1 mRNA expression was upregulated following incubation with 10^{-7} mol/L ET-1 for 30 min (188.5 ± 18.0%, $n = 8$, $p \leq 0.05$, Fig. 2) and 60 min (212.5 ± 24.1%, $n = 7$, $p \leq 0.01$). After prolonged ET-1 stimulation SGK1 mRNA expression, however, gradually declined to control levels (90 min: 144.2 ± 61.4%; 120 min: 88.17 ± 10.6%; 180 min: 115.5 ± 2.9%; 240 min: 121.8 ± 29.2%; $n = 3$, $p > 0.05$). The preincubation of the aortic rings with the ET_A receptor antagonist LU 302146 or the ET_A/ET_B receptor antagonist LU 302872 prior to the addition of ET-1 (10^{-7} mol/L) for 60 min prevented this increase in SGK1 mRNA (LU 302146: 143.1 ± 30.9%, $n = 4$, $p > 0.05$ versus control; LU 302872: 117.1 ± 26.14%, $n = 4$, $p > 0.05$ versus control). Neither the ET_A nor the ET_A/ET_B receptor antagonist alone had any effect on SGK1 mRNA expression (LU 302146: 122.0 ± 22.9%, $n = 3$, $p > 0.05$ versus control; LU 302872: 137.9 ± 44,8%, $n = 3$, $p > 0.05$ versus control).

3.3. Interaction of the aldosterone and ET-1 system

Both ET-1 and aldosterone are important regulators of vasoconstriction and might regulate each other's expression. In order to investigate whether aldosterone affected ET-1 levels, pre–pro ET-1 gene transcript levels were determined. After treatment of smooth muscle cells for 60–180 min with aldosterone pre–pro ET-1 mRNA levels were significantly increased (Fig. 3). This induction was prevented by co-incubation of aldosterone (120 min) with the mineralocorticoid antagonist spironolactone. Incubation with aldosterone for 240 min, 300 min or 360 min reduced the mRNA transcripts to normal levels. In contrast, ET-1 did apparently not affect aldosterone expression, even when A-10 cells were incubated for up 3 days with ET-1 (10^{-7} mol/L). Neither was aldosterone measurable in the culture medium nor was aldosterone synthase mRNA expression detectable after ET-1 stimulation (data not shown).

3.4. Combined effects of ET-1 and aldosterone on SGK1 expression

To determine a possible additive effect of ET-1 and aldosterone on SGK1 mRNA expression, smooth muscle cells were incubated with either ET-1 (10^{-7} mol/L) or aldosterone (10^{-7} mol/L) or with both hormones together. Co-incubation of both agonists did not result in an additional increase in SGK1 gene expression in comparison to the treatment of cells with either hormone alone (Fig. 4). The ET_A receptor antagonist LU 302146 (126 ± 7.2%, $n = 6$, p = n.s.) and spironolactone (104 ± 5.9%, $n = 6$, p = n.s.) alone did not significantly influence SGK1 mRNA levels. Moreover, the aldosterone-induced SGK1 increase was not significantly impaired by the ET_A inhibitor LU 302146, whereas the minerolocorticoid antagonist spironolactone abolished SGK1 mRNA expression to baseline levels (Fig. 4). In contrast, spironolactone could not inhibit the ET-1 mediated increase in SGK1 mRNA. This result therefore indicates that ET-1 and aldosterone independently increase SGK1 mRNA expression.

We further investigated the effect of aldosterone and ET-1 on the protein levels of SGK1. Densitometric analysis of Western blots revealed that both hormones induced a more than 6-fold induction of SGK1 expression (Fig. 5). SGK1 protein amounts in vascular smooth muscle cells were significantly increased following 3 h of incubation with ET-1 (660.1 ± 55.0%, $n = 8$, $p < 0.01$) or aldosterone (641.8 ± 224%, $n = 3$, $p < 0.01$). Consistent with the previous experiments, co-incubation of ET-1 and aldosterone showed an increase of SGK1 protein comparable to treatments with either hormone alone (635.5 ± 70.2%, $n = 3$, $p < 0.01$). As expected, pretreatment of the cells with the ET_A receptor antagonist prevented the ET-1 mediated increase of SGK1 protein (135.4 ± 28.2%, $n = 3$, n.s.), while spironolactone inhibited the stimulatory effect of aldosterone (142 ± 36.2%, $n = 3$, n.s.).

3.5. Myocardial SGK1 mRNA expression in subtotally nephrectomized rats

In order to explore a possible in vivo significance of the findings obtained with isolated smooth muscle cells, we next

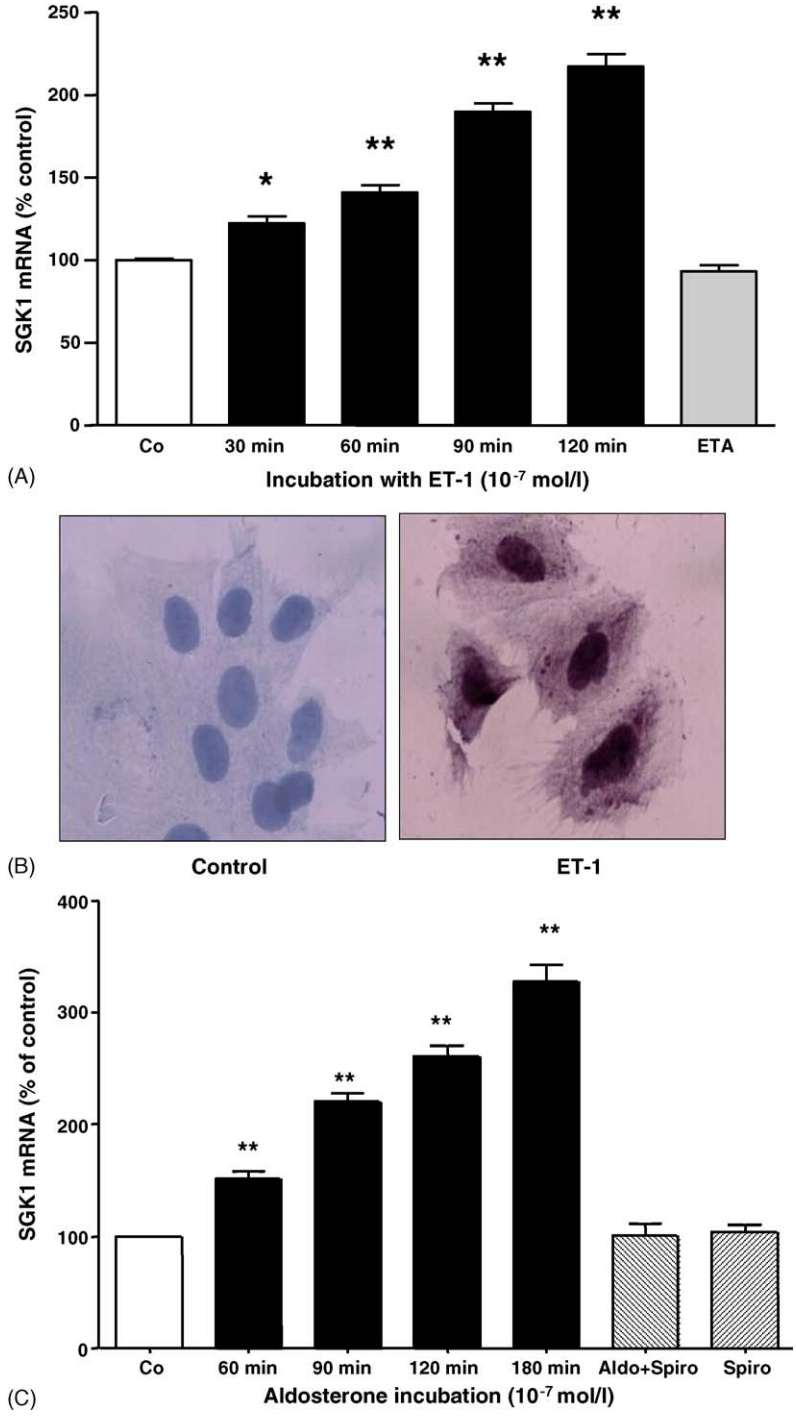

Fig. 1 – ET-1 increases SGK1 mRNA expression. (A) Incubation of A-10 smooth muscle cells with ET-1. Cells were incubated with ET-1 (10^{-7} mol/L) for the indicated time, before total mRNA was isolated. SGK1 expression was measured by real time RT-PCR. ET-1 induced a time-dependent increase in SGK1 mRNA transcripts, which was abolished by the ET_A receptor antagonist LU 302146 (ET_A). Shown are the effects in mean ± S.E.M. of three to six experiments. Statistical significance $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control. (B) In situ hybridization of SGK1 mRNA in A-10 cells. Hybridization of untreated control cells (left panel) with antisense SGK1 showed hardly any hybridization signals. After 1 h of ET-1 incubation the cells revealed a strong increase of SGK1 transcripts (right panel). (C) Effect of aldosterone. SGK1 expression was measured by real-time RT-PCR after incubation of A-10 cells with alosterone (10–7 mol/L) for the indicated time. Shown are the effects in mean ± S.E.M. of three to six experiments. Statistical significance $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control.

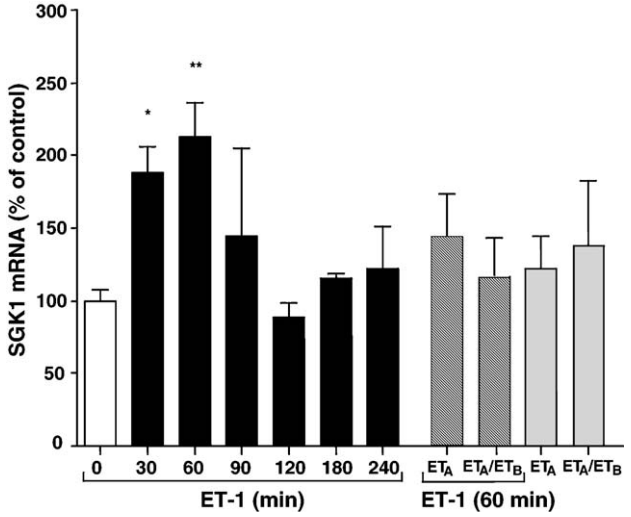

Fig. 2 – Effect of ET-1 on SGK1 mRNA expression in aortic rings. Thoracic aortas were isolated and incubated for the indicated time with ET-1 (10^{-7} mol/L), before total mRNA was isolated. The ET_A and ET_{AB} receptor antagonists LU 302146 and LU302872, respectively, were added 30 min, before the aortic rings were stimulated with ET-1 for further 60 min. ET-1 induced a time-dependent increase in mRNA of SGK1. ET receptor blockade inhibited the ET-1 mediated increase in SGK1 mRNA. The results show the mean ± S.E.M. of three to eight experiments. Statistical significance $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control.

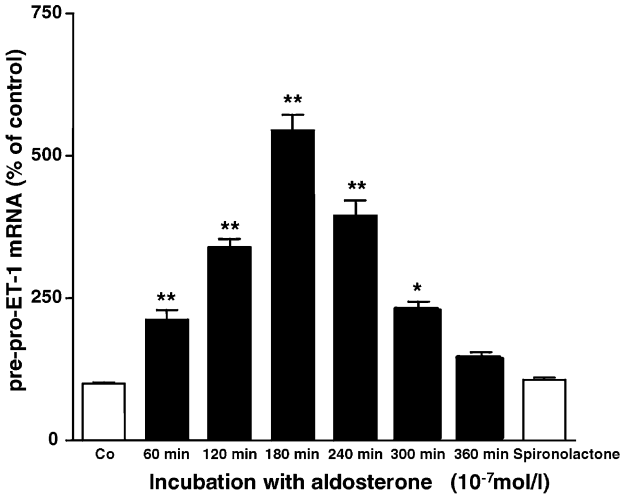

Fig. 3 – Effect of aldosterone on ET-1 expression. A-10 smooth muscle cells were incubated with aldosterone (10^{-7} mol/L) for 60–360 min. Following RNA isolation pre–pro ET-1 mRNA levels were assessed by real-time RT-PCR. Aldosterone induced a time-dependent increase in SGK1 mRNA expression that was abolished by the mineralocorticoid antagonist spironolactone (120 min, 10^{-7} mol/L). Shown are the means ± S.E.M. of three to six experiments. Statistical significance $^{**}p < 0.01$ vs. control, Statistical significance $^{*}p < 0.05$ vs. control.

employed an in vivo model with increased ET-1 concentrations. To this end, rats were subtotally nephrectomized animals resulting in ET-1 induction, and thereafter SGK1 expression was analyzed in the myocardium of the left ventricle. Twelve weeks after nephrectomy myocardial ET-1 concentrations were significantly increased (controls: 3.92 ± 0.33 pg/g tissue, $n = 5$; SNX: 7.76 ± 0.84 pg/g tissue, $n = 5$, $p < 0.01$; ET_A LU 302146: 6.89 ± 0.54 pg/g tissue, $n = 5$, p = n.s.). Importantly, as assessed by real time RT-PCR analysis of myocardial tissues, the increased myocardial levels of ET-1 were associated with a strong increase of SGK1 expression. In comparison to the controls, in SNX animals SGK1 mRNA was increased to 209.5 ± 8.6% ($n = 3$, $p < 0.01$, Fig. 6). The increase of SGK1 transcript levels following subtotal nephrectomy was almost completely prevented by the ET_A antagonist LU 302146 (30 mg/kg bw/d) (118.8 ± 4.4%, $n = 3$, p = n.s.). Thus, these data provide strong evidence that increased ET-1 levels following subtotal nephrectomy lead to subsequent upregulation of SGK1 expression in vivo.

4. Discussion

The present study demonstrates for the first time that ET-1 is an effective activator of SGK1 mRNA expression. More importantly, our data also show that stimulation of SGK1 expression occurs not only in vitro in A-10 cells and freshly isolated aortic rings, but also in an animal model of subtotally nephrectomized rats that have chronically increased ET-1 levels [37]. The stimulatory effect of ET-1 on SGK1 mRNA expression was in a similar range as observed after treatment with aldosterone, a known activator of SGK1 expression. These findings therefore suggest that, similar to aldosterone,

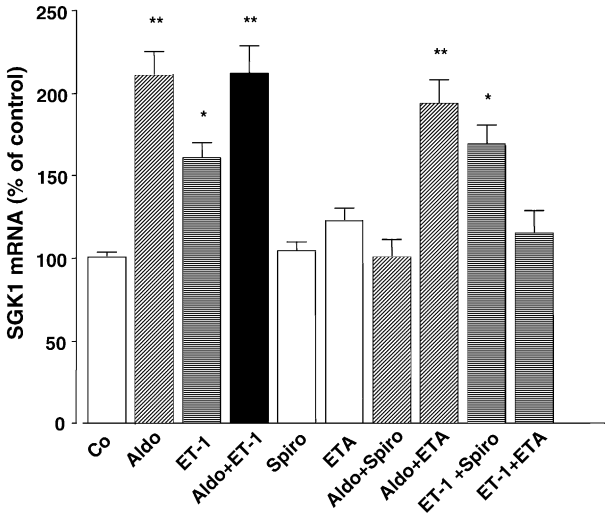

Fig. 4 – Comparison of the effects of aldosterone and ET-1 on SGK1 mRNA expression. A-10 cells were incubated with aldosterone (Aldo, 10^{-7} mol/L) and/or ET-1 (10^{-7} mol/L) in the presence or absence of spironolactone (Spiro, 10^{-7} mol/L) or the ET_A receptor antagonist LU 302146 (ET_A, 10^{-6} mol/L). After 3 h of incubation, SGK1 expression was quantified by real-time RT-PCR analysis. Shown are means ± S.E.M. of three to eight experiments. Statistical significance $^{**}p < 0.01$ vs. control.

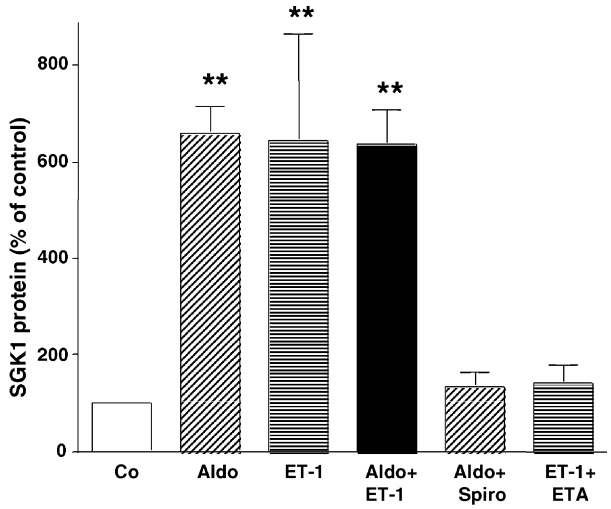

Fig. 5 – Effect of ET-1 and aldosterone on SGK1 protein expression. A-10 cells were incubated in presence and absence of aldosterone (Aldo) or ET-1 (each 10^{-7} mol/L) or a combination of both agents. After 3 h cell lysates were prepared and subjected to Western blot analysis. Expression of SGK1 was quantified by densitometric analysis of the blots and is given as the percentage of control. Shown are means ± S.E.M. of three to eight experiments. Statistical significance $^{**}p < 0.01$ vs. control. The upper panel shows a representative immunoblot.

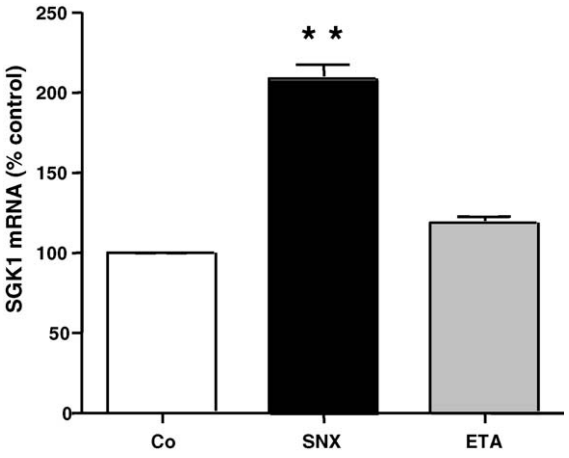

Fig. 6 – Myocardial SGK1 mRNA expression in subtotally nephretomized rats. Rats were subtotally nephrectomized (SNX) or sham operated (controls, Co). One group of animals was then further left untreated (SNX), and the other nephrectomized rats were treated with the ET_A receptor antagonist LU 302146 (30 mg/kg bw) for 12 weeks. Afterwards myocardial tissues were examined, and SGK1 gene transcripts were measured by real-time RT-PCR. In SNX animals ($n = 3$) SGK1 gene transcription was increased in comparison to controls ($n = 3$). Administration of the ET_A receptor antagonist prevented this increase in SGK1 gene transcription. Shown are means ± S.E.M. of three experiments. Statistical significance $^{**}p < 0.01$ vs. control.

at least part of the blood-pressure elevating effects of ET-1 might be mediated by SGK1.

Up to now, several growth factors and stress stimuli have been found to transcriptionally activate SGK1 expression, but the signalling pathways that mediate the activation of SGK1 gene transcription by different agonists have not yet been studied extensively. Increased transcription of the SGK1 gene in response to osmotic stress but not to glucocorticoids appears to be mediated by stress-activated protein kinase p38 [20,21], whereas gene transcription induced by follicle-stimulating hormone might also require cAMP [22]. Interestingly, in our experiments ET-1 and aldosterone had no additive effects on SGK1 mRNA and protein expression. Thus, aldosterone and ET-1 might stimulate SGK1 mRNA expression through similar signal transduction pathways. Alternatively, expression of SGK1 might trigger a negative feedback loop, which limits further upregulation of SGK1 transcription. On the other side, the ET_A antagonist and spironolactone were not able to completely suppress SGK1 mRNA expression, indicating that in A-10 cells SGK1 transcripts are constitutively present at low basal levels. Like other known inducers of SGK1, also ET-1 induced a rapid and transient increase of SGK1 mRNA transcription, which resembled immediate/early gene expression and followed a similar kinetic as reported for other stimuli, such as luteinizing and follicle-stimulating hormone, dexamethasone or TGF-β [11,19,38]. In A-10 cells, both aldosterone and ET-1 induced an approximately 2-fold increase of SGK1 mRNA transcription. In other cell types including rat colon epithelial cells, renal cortex cells or A6 kidney cells, aldosterone has been reported to induce a 2–10-fold increase in SGK1 mRNA [11,39,40]. It is tempting to speculate that this slightly different inducibility of SGK1 expression is due to the use of different experimental systems and means of mRNA quantification.

Both aldosterone and ET-1 have been implicated in the development of salt-sensitive hypertension [2–5,16]. It is conceivable that SGK1 may contribute to both aldosterone- and ET-1-dependent signalling in blood pressure control. In gene-targeted mice lacking functional SGK1 renal salt retention is impaired, and salt-deficient diet leads to renal salt loss and a subsequent decline of blood pressure [41]. Conversely, a presumed gain-of-function polymorphism of the SGK1 gene in twins was associated with increased blood pressure values [30]. Hyperactivity of SGK1 might therefore be a cause of high blood pressure.

Several observations indicate that the hypertensive action of ET-1 is at least in part due to an increase of vascular reactivity. Incubation of human mammary artery rings with low concentrations of ET-1 potentiated serotonin- and norepinephrine-mediated vasoconstriction [42]. In salt-sensitive hypertensive rats, high sodium intake was associated with exaggerated vascular reactivity in response to vasoconstrictory stimuli, which may contribute to an increase in blood pressure [43]. There is also evidence that ET-1-induced hypertension is dependent on the salt uptake. For instance, Mortensen and co-workers demonstrated a normal blood pressure in animals after 2 weeks of ET-1 infusion and administration of a low-salt diet. However, an increased sodium content in the drinking water resulted in significant

hypertension, indicating the salt sensitivity of ET-1-induced hypertension [44,45]. Since SGK1 is thought to be responsible for the "fine tuning" of sodium balance, ET-1 might regulate salt-sensitive hypertension via an induction of SGK1-dependent processes, analogously to the aldosterone system [11–15]. In this pathway, SGK1 regulates surface expression of the renal epithelial sodium channel ENaC through the phosphorylation-induced inhibition of Nedd4, an ubiquitin ligase involved in ENaC degradation [46].

It has been reported that in the model of subtotal nephrectomy hypertension developed at normal salt diet, but not at low sodium uptake [37]. Using a similar animal system, we found that myocardial ET-1 levels were elevated, an effect presumably contributing to or accounting for the increase of myocardial SGK1 expression. More important and underscoring our finding is the fact that a blockade of ET_A receptors completely prevented this increase in SGK1 mRNA expression.

Whether the pathogenesis of cardiac hypertrophy that is associated with the experimental model of subtotal nephrectomy also involves elevated SGK1 expression, remains to be clarified. A striking increase of SGK1 mRNA expression has been observed in a variety of fibrosing diseases including diabetic nephropathy, glomerulonephritis, hepatic cirrhosis, pulmonary fibrosis, Crohn's disease and fibrotic pancreatitis [19,24,25,31,47–49]. Thus, SGK1 may be involved in the stimulation of extracellular matrix deposition. As shown in our experiments, SGK1 mRNA expression was inhibited by ET_A receptor blockade. This phenomenon might therefore contribute to the beneficial effects of ET_A blockers in uremia [50,51]. In conclusion, the present study discloses a stimulating effect of ET-1 on the expression of SGK1 both in vitro and in vivo. This stimulating effect might participate in ET-1-dependent regulation of extracellular volume, blood pressure and cardiac modelling.

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