Up-Regulation of FGF23 Release by Aldosterone

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Abstract

The fibroblast growth factor (FGF23) plasma level is high in cardiac and renal failure and is associated with poor clinical prognosis of these disorders. Both diseases are paralleled with hyperaldosteronism. Excessive FGF23 levels and hyperaldosteronism are further observed in Klotho-deficient mice. The present study explored a putative aldosterone sensitivity of Fgf23 transcription and secretion and the putative involvement of the aldosterone sensitive serum & glucocorticoid inducible kinase SGK1 and SGK1 sensitive transcription factor NFκB and store operated Ca^{2+} entry (SOCE). Serum FGF23 levels were determined by ELISA in mice following sham treatment or exposure to deoxycorticosterone acetate (DOCA) or salt depletion. In osteoblastic UMR106 cells transcript levels were quantified by qRT-PCR, cytosolic Ca^{2+} concentration utilizing Fura-2-fluorescence, and SOCE from Ca^{2+} entry following store depletion by thapsigargin. As a result, DOCA treatment and salt depletion of mice elevated the serum C-terminal FGF23 concentration. In UMR106 cells aldosterone enhanced and spironolactone decreased SOCE. Aldosterone further increased Fgf23 transcript levels in UMR106 cells, an effect reversed by mineralocorticoid receptor blockers spironolactone and eplerenone, SGK1 inhibitor EMD638683, NFκB-inhibitor withaferin A, and Ca^{2+} channel blocker YM58483. In conclusion, Fgf23 expression is up-regulated by aldosterone, an effect sensitive to SGK1, NFκB and store-operated Ca^{2+} entry.

Key words:

1,25(OH)₂D₃; mineralocorticoid, spironolactone, SOCE, calcium, Orai1, NFκB

Introduction

Regulation of calcium phosphate metabolism involves FGF23 (fibroblast growth factor 23), which is mainly released from bone cells [1]. FGF23 decreases formation and fosters inactivation of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) by down-regulating renal 1 α hydroxylase (Cyp27b1) and up-regulating 25-hydroxyvitamin D 24-hydroxylase (Cyp24a1) [2]. FGF23 thus lowers serum levels of 1,25(OH)₂D₃ [3,4], which stimulates renal and intestinal phosphate and calcium transport[5,6]. FGF23 deficiency is followed by increase of plasma 1,25(OH)₂D₃ levels, hyperphosphatemia, and hypercalcemia leading to vascular calcification, rapid aging and a substantial decrease of the life span [2]. Vascular calcification is stimulated by hyperphosphatemia [7], a predictor of mortality [8] and by aldosterone [9], which similarly impacts on life span [9].

Excessive plasma FGF23 levels are observed and are associated with accelerated disease progression, morbidity and/or mortality in several clinical disorders including cardiac failure [10,11], acute renal failure [12], chronic kidney disease [11,13,14], diabetic nephropathy [15] and hepatic failure [16]. The pathophysiological significance of enhanced FGF23 formation, has, however, remained ill-defined [17,18] and little is known about mechanisms accounting for the up-regulation of FGF23 release in these clinical disorders.

Most recently, Fgf23 transcription has been shown to be stimulated by an increase in the cytosolic Ca²⁺ concentration ([Ca²⁺]_i) secondary to upregulation of store operated Ca²⁺ entry (SOCE) [19], which is triggered by depletion of intracellular Ca²⁺ stores [20]. SOCE is accomplished by the calcium release-activated channel (CRAC) moiety Orai1 (CRACM1) [21], which is activated by stromal interaction molecule 1 (STIM1) [22]. Expression and activity of Orai1 are up-regulated by the transcription factor NF κ B [23].

Regulators of NF κ B include mineralocorticoids [24,25]. In patients with non-ischemic cardiac disease and early chronic kidney disease high FGF23 plasma levels are associated with high plasma aldosterone concentrations [11].

The present study explored whether aldosterone modifies FGF23 release and, if so, whether the effect involves NF κ B and Ca²⁺ entry.

Materials and Methods

Animal experiments

The animal experiments were conducted according to the German law for the welfare of animals and were approved by the state of Baden-Württemberg (Regierungspräsidium Tübingen). Fifty μ l blood was collected from male and female C57BL/6 mice (8-20 weeks old). Then, mice were sham-treated or treated with a single s.c. injection of deoxycorticosterone acetate (DOCA; 100 mg/kg b.w.; Sigma, Schnelldorf, Germany) or spironolactone (75 mg/kg b.w.; Sigma). Twelve hours after the injection, another 50 μ l blood was collected. For the effect of low salt diet on FGF23 release, male and female mice (2-13 months old) were fed a control diet or a low salt diet (containing <0.2% Na⁺ and Cl⁻; Altromin, Lage, Germany) for a total of 7-14 days and subsequently 50 μ l blood were collected. Serum C-terminal FGF23 was determined with an ELISA kit (Immutopics, San Clemente, USA). Serum aldosterone and corticosterone were quantified by means of kits from IBL (Hamburg, Germany) according to the manufacturers' protocol.

Cell culture

UMR106 rat osteosarcoma cells were cultured in DMEM high glucose medium supplemented with 10% FCS and 1% penicillin/streptomycin under standard culture

conditions. Cells were pretreated with 100 nM 1,25(OH)₂D₃ (Sigma) for 42 h. Then, 100 nM aldosterone (Sigma) was added without or with 10 μM spironolactone (Sigma), 10 μM eplerenone (Sigma), 100 nM YM58483 (TOCRIS), 500 nM withaferin A (TOCRIS), or 50 μM SGK1 inhibitor EMD638683 (Merck Darmstadt, Germany) for 6 h.

For the calcium measurements and the quantification of *Orai1* transcripts, cells were treated with 100 nM aldosterone (Sigma) or 10 μM spironolactone (Sigma) for 24 h in serum-free medium.

Quantification of mRNA expression

For the mRNA expression analysis in UMR106 cells, the final volume of the qRT-PCR reaction mixture was 20 μl and contained: 2 μl cDNA, 1 μM of each primer, 10 μl GoTaq Master Mix (Promega), and sterile water up to 20 μl. PCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 58°C for 30 s. Quantitative RT-PCR was performed on a BioRad iCycler iQTM Real-Time PCR Detection System (Bio-Rad Laboratories, Munich, Germany).

For the determination of *Fgf23* transcripts in mouse bone, bone was homogenized in liquid nitrogen using mortar and pestle. Total mRNA from bone was extracted with TRIzol (Invitrogen, Switzerland) followed by purification with RNeasy Mini Kit (Qiagen, Switzerland) according to the manufacturer's protocol. DNase digestion was performed using the RNase-free DNase Set (Qiagen, Switzerland). Total RNA extractions were analyzed for quality, purity, and concentration using the NanoDrop ND-1000 spectrophotometer (Wilmington, Germany). RNA samples were diluted to a final concentration of 100 ng/μl and cDNA was prepared using the TaqMan Reverse Transcriptase Reagent Kit (Applied Biosystems, Roche, Foster City, CA). In brief, in a reaction volume of 40 μl, 300 ng of RNA was used as template and mixed with the following final concentrations of RT buffer (1x): MgCl₂ (5.5 mmol/l), random hexamers (2.5 μmol/l), dNTP mix (500 μmol/l each), RNase inhibitor (0.4 U/μl), multiscribe reverse transcriptase (1.25 U/μl), and RNase-free water. Reverse transcription was performed with thermocycling conditions of 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min on a thermocycler (Biometra, Göttingen, Germany). Semi-quantitative real-time PCR (RT-PCR) was performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Primers were chosen to spanning intron–exon boundaries to exclude genomic DNA contamination. Probes were labeled with the reporter dye FAM at the 5'-end and the quencher dye TAMRA at the 3'-end (Microsynth, Balgach, Switzerland). Real-time PCR reactions were performed using KAPA PROBE FAST qPCR Kit (Kapa Biosystems, USA).

The following primers were used:

Rat Tbp (TATA box-binding protein):

forward (5'-3'): ACTCCTGCCACACCAGCC

reverse (5'-3'): GGTCAAGTTTACAGCCAAGATTCA

Rat Fgf23

forward (5'-3'): TGGCCATGTAGACGGAACAC

reverse (5'-3'): GGCCCCTATTATCACTACGGAG

Rat Orai1

forward (5'-3'): CGTCCACAACCTCAACTCC

reverse (5'-3'): AACTGTCGGTCCGTCTTAT

Mouse Fgf23

forward (5'-3'): TCGAAGGTTTCCTTTGTATGGAT

reverse (5'-3'): AGTGATGCTTCTGCGACAAGT

Calculated mRNA expression levels were normalized to the expression levels of *Tbp* (in rat derived cell lines) or *HPRT/18S* (in mice) of the same cDNA sample. Relative quantification of gene expression was performed using the $\Delta\Delta C_t$ method.

Measurement of intracellular Ca^{2+}

To determine the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), UMR106 cells were loaded with Fura-2/AM (2 μM , Molecular Probes, Göttingen, Germany) for 15 min at 37°C. Fluorescence measurements were carried out with an inverted phase-contrast microscope (Axiovert 100, Zeiss, Oberkochen, Germany). Cells were excited alternatively at 340 or 380 nm and the light was deflected by a dichroic mirror into either the objective (Fluar 40 $\times$ /1.30 oil, Zeiss, Oberkochen, Germany) or a camera (Proxitronic, Bensheim, Germany). Emitted fluorescence intensity was recorded at 505 nm. Data acquisition was accomplished by using specialized computer software (Metafluor, Universal Imaging Downingtown, USA). As a measure for the increase in the cytosolic Ca^{2+} concentration, the slope and peak of the changes in the 340/380 nm ratio were determined in each experiment.

To determine SOCE, intracellular Ca^{2+} was measured before and after removal of extracellular Ca^{2+} (and addition of 0.5 mM EDTA), followed by addition of thapsigargin (1 μM) and subsequent re-addition of extracellular Ca^{2+} to Ringer solution, composed of (in mM): 125 NaCl, 5 KCl, 1.2 MgSO_4 , 32.2 HEPES (NaOH/pH 7.4), 2 Na_2HPO_4 , 0 or 2 CaCl_2 and 0.5 or 0 EGTA, respectively, and 5 glucose.

Immunofluorescence

UMR106 cells treated with 100 nM aldosterone were cultured on 4-well chamber slides (Thermo scientific), washed, and fixed with 4% paraformaldehyde for 30 min at room temperature. For blocking unspecific binding, UMR106 cells were incubated with 3% Albumin Fraction V (Carl Roth, Karlsruhe, Germany), 5% normal goat serum (Sigma, Schnellendorf, Germany), and 0.5% Triton in PBS (PAA, Cölbe, Germany) for 30 min at room temperature. Then, the cells were exposed to rabbit anti-p65 (1:1000, Genetex, Irvine, USA) at 4°C in a humidified chamber overnight. The cells were rinsed four times with PBS and incubated with DyLight® 488-conjugated goat anti-rabbit antibody (1:3000, BIOZOL, Eching, Germany) for 1 h at room temperature. After four washing steps the nuclei were stained with DRAQ-5 dye (1:400; BIOZOL) for 30 min at room temperature. The slides and coverslips were mounted with FluorSave™ Reagent (Calbiochem, Darmstadt, Germany). Images were taken on a LSM 5 EXCITER confocal laser scanning microscope (Zeiss, Germany) with a water-immersion Plan-Neofluar 40 $\times$ /1.3 NA differential interference contrast and analyzed with the instrument's software.

Statistics

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

Results

In order to test for an effect of mineralocorticoids on FGF23 release, a single dose of DOCA (100 mg/kg b.w. s.c.) or vehicle was injected into wild type mice. Twelve hours later the serum C-terminal FGF23 (cFGF23) level was determined. As illustrated in Fig. 1A, the injection of DOCA was followed by a marked and significant increase in the serum cFGF23 concentration. In a second series of experiments, mice were fed a low salt diet. As expected, this diet induced an elevation of the serum aldosterone level (889 ± 217 pg/ml; $n=5$) compared to standard chow-fed mice (268 ± 27 pg/ml; $n=5$; $p<0.01$). The diet did not significantly affect the serum corticosterone level (low salt diet: 162 ± 32 ng/ml; $n=5$; standard chow: 120 ± 31 ng/ml; $n=5$). As illustrated in Fig 1B, the low salt diet also elevated the serum concentration of

C-terminal FGF23. The main site of FGF23 formation is the bone. Accordingly, we found a higher level of Fgf23 transcripts in bone from DOCA-treated mice compared to untreated mice (Fig. 1C). Treatment with spironolactone (12 h, 75 mg/kg b.w.) did, however, not significantly influence the serum FGF23 concentration (control: 201 ± 6 pg/ml, n=5; spironolactone: 332 ± 65 pg/ml, n=5). Similarly, spironolactone treatment did not block the increase in serum FGF23 induced by low-salt diet (serum FGF23 before low-salt diet: 134 ± 15 pg/ml; serum FGF23 after two weeks of low-salt diet: 194 ± 13 pg/ml; n=8; $p < 0.01$).

Fluorescence optics was employed to test whether aldosterone modifies the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Store-operated Ca^{2+} entry (SOCE) requires depletion of intracellular Ca^{2+} stores which was accomplished by inhibition of the sarcoendoplasmic Ca^{2+} ATPase (SERCA) with thapsigargin (1 μM) in the absence of extracellular Ca^{2+} . SOCE was estimated from the increase in $[\text{Ca}^{2+}]_i$ following re-addition of extracellular Ca^{2+} . UMR106 cells were treated with aldosterone or the mineralocorticoid receptor antagonist spironolactone and intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) were determined prior to and following removal of extracellular Ca^{2+} , addition of thapsigargin, and re-addition of extracellular Ca^{2+} . As illustrated in Fig. 2A-C, aldosterone did not significantly modify the thapsigargin-induced increase in $[\text{Ca}^{2+}]_i$ but significantly enhanced the elevation of $[\text{Ca}^{2+}]_i$ following re-addition of extracellular Ca^{2+} . The aldosterone antagonist spironolactone did not significantly modify the thapsigargin-induced increase in $[\text{Ca}^{2+}]_i$, but significantly blunted the increase in $[\text{Ca}^{2+}]_i$ following re-addition of extracellular Ca^{2+} (Fig. 2D-F).

A further series of experiments explored whether the influence of aldosterone and spironolactone on Ca^{2+} signaling in UMR106 cells was involved in the transcriptional regulation of Fgf23. Aldosterone (Fig. 3A) significantly elevated the Orai1 transcript level in UMR106 cells. Moreover, aldosterone up-regulated the transcription of Fgf23, an effect virtually abrogated by spironolactone, the Orai inhibitor YM58483, or the NF κ B inhibitor withaferin A (Fig. 3B). The dose-response curve of the aldosterone effect on FGF23 is shown in Fig 3C. The aldosterone antagonist eplerenone similarly reversed the aldosterone effect on Fgf23 (Fig. 3D). Immunofluorescence confirmed activation of NF κ B by aldosterone in UMR106 cells (Fig. 3E).

A possible mediator of the aldosterone effect on Orai1 is SGK1 which has been shown to up-regulate this channel. Employing qRT-PCR we indeed found a positive effect of aldosterone on the Sgk1 transcript level in UMR106 cells (control: 0.73 ± 0.04 a.u., n=12; 100 nM aldosterone: 1.02 ± 0.05 a.u., n=12; $p < 0.001$). Moreover, aldosterone failed to stimulate Fgf23 expression in the presence of a SGK1 inhibitor (Fig. 3F).

The dose effects of mineralocorticoid antagonism with spironolactone or eplerenone are displayed in Fig. 4A and 4B, respectively. Notably, the effect of higher aldosterone concentrations on FGF23 transcription were also blocked by the glucocorticoid antagonist mifepristone (Fig. 4C).

Discussion

The present observations reveal that mineralocorticoids may contribute to the regulation of Fgf23 transcription and release *in vivo*. They further shed some light on the cellular mechanisms involved. According to previous observations [19] FGF23 release is stimulated by an increase in the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), which could be triggered by store operated Ca^{2+} entry (SOCE). Aldosterone increased and spironolactone decreased SOCE and thus modified transcription of Fgf23. SOCE is mediated by isoforms of the channel Orai (CRAC) [21], which are activated upon store depletion by STIM1 and STIM2 [22]. UMR106 cells express high levels of Orai1 and STIM2 [19]. The expression of Orai1 is stimulated by the transcription factor NF κ B [23], which thus impacts on Fgf23 expression [19]. Aldosterone has previously been shown to up-regulate NF κ B [24,26]. The present study reveals that the effect of aldosterone on Fgf23

transcript levels is abrogated by NFκB inhibitors. The present observations do, however, not rule out that NFκB plays a permissive role in the regulation of FGF23 expression and release.

As aldosterone is effective at concentrations as low as 1 nM, the effect of aldosterone is most likely - at least in part - due to stimulation of mineralocorticoid receptors. The effect of higher aldosterone concentrations is, however, blunted by the glucocorticoid antagonist mifepristone, an observation pointing also to an involvement of glucocorticoid receptors. Noteworthy, both, mineralocorticoid and glucocorticoid receptors upregulate the serum & glucocorticoid inducible kinase (SGK1) which has been shown to induce Orai1-dependent Ca^{2+} entry by activating NFκB [27]. Along those lines, pharmacological SGK1 inhibition reversed the effect of aldosterone on Fgf23 expression. Spironolactone treatment, however, did not reverse the increase in serum FGF23 induced by low-salt diet. Thus, the possibility must be kept in mind that mechanisms other than mineralocorticoid receptor activation contribute to the up-regulation of FGF23 release during dehydration. Along those lines, renal klotho expression is not only down-regulated by mineralocorticoids but as well by ADH [28].

Chronic kidney disease [29-31], heart failure [32-34], diabetic nephropathy [35], and hepatic failure [34] are all characterized by hyperaldosteronism. According to the present observations, the high FGF23 plasma concentration in heart failure [10,11], acute renal failure [12], chronic kidney disease [11,13,14], diabetic nephropathy [15] and hepatic failure [16] could at least in part be secondary to the hyperaldosteronism associated with these diseases. The effect of aldosterone may at least partially be mediated by the serum & glucocorticoid inducible kinase SGK1, which is up-regulated by aldosterone and during diabetes [36] and is known to up-regulate Orai1 [23].

In conclusion, aldosterone is a powerful stimulator of FGF23 formation *in vitro* and *in vivo*. Aldosterone is at least partially effective by NFκB dependent up-regulation of Orai1 with subsequent enhancement of store operated Ca^{2+} entry.

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Figure Legends

Fig. 1. Effect of aldosterone on the FGF23 serum level. **A.** Arithmetic means $\pm$ SEM (n=9-11) of the serum FGF23 concentration in wild type mice sham-treated (white bar) or treated with a single DOCA injection (100 mg/kg b.w. s.c.) for 12 hrs (black bar). **B.** Arithmetic means $\pm$ SEM (n=11) of the serum FGF23 concentration in wild type mice treated with normal diet (white bar) or with low salt diet for 7-14 days (black bar). **C.** Arithmetic means $\pm$ SEM (n=4-5) of the relative Fgf23 mRNA abundance in wild type mice sham-treated (white bar) or treated with a single DOCA injection (100 mg/kg b.w. s.c.) for 12 hrs (black bar). *(p<0.05), **(p<0.001), *** (p<0.001), indicate significant difference.

Fig. 2. Effect of aldosterone and spironolactone on SOCE. **A,D.** Representative original tracings showing intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) in Fura-2/AM loaded UMR106 cells prior to and following removal of extracellular Ca^{2+} , addition of the sarco-endoplasmic Ca^{2+} ATPase (SERCA) inhibitor thapsigargin (1 μM) and readdition of extracellular Ca^{2+} , all in the absence (open circles) and presence (closed circles) of aldosterone (**A**, 100 nM, 24 h), or spironolactone (**D**, 10 μM , 24 h). **B,C,E,F.** Arithmetic means $\pm$ SEM of the peak (left) and slope (right) values of the $[\text{Ca}^{2+}]_i$ increase following addition of thapsigargin reflecting Ca^{2+} release from intracellular stores (**B,E**) and of $[\text{Ca}^{2+}]_i$ increase following readdition of extracellular Ca^{2+} reflecting store operated Ca^{2+} entry (**C,F**) in UMR106 cells incubated without (white bars) or with (black bars) aldosterone (**B-C**, 100 nM, 24 h, n=72-80), or spironolactone (**E-F**, 10 μM , 24 h, n=121-133).*(p<0.05), *** (p<0.001) indicate significant difference (student's t-test).

Fig.3. Effect of aldosterone on Fgf23 transcript levels in UMR106 cells with or without spironolactone, eplerenone, YM58483 and withaferin A. **A.** Arithmetic means $\pm$ SEM (n=18) of Orai1 mRNA abundance (relative to Tbp mRNA) in UMR106 cells incubated without (white bar) or with (black bar) aldosterone (100 nM, 24 h). **B.** Arithmetic means $\pm$ SEM (n=7) of Fgf23 mRNA abundance (relative to Tbp mRNA) in UMR106 cells incubated without (white bars) or with (black bars) aldosterone (100 nM, 6 h) in the absence (left bars) or presence (right bars) of spironolactone (10 μM) of Orai inhibitor YM58483 (100 nM) or of NF κ B inhibitor withaferin A (500 nM). **C.** Arithmetic means $\pm$ SEM (n=36-37) of Fgf23 mRNA abundance (relative to Tbp mRNA) in UMR106 cells incubated without (white bars) or with (black bars) aldosterone (6 h) at the indicated concentration. **D.** Arithmetic means $\pm$ SEM (n=11) of Fgf23 mRNA abundance (relative to Tbp mRNA) in UMR106 cells incubated without (white bars) or with (black bars) aldosterone (100 nM, 6 h) in the absence (left bars) or presence (right bars) of eplerenone (10 μM). **E.** Original immunofluorescence images demonstrating nuclear staining (Draq5; Red; left images), p65-specific staining (NF κ B; Green middle images), and an overlay of both, nuclear and p65-specific staining (right images) in UMR106 cells incubated without (upper images) or with (lower images) aldosterone (100 nM, 24 h) Scale bar: 20 μm . **F.** Arithmetic means $\pm$ SEM (n=12) of Fgf23 mRNA abundance (relative to Tbp mRNA) in UMR106 cells incubated without (white bars) or with (black bars) aldosterone (100 nM, 6 h) in the absence (left bars) or presence (right bars) of SGK1 inhibitor EMD638683 (50 μM). *, **, *** (p<0.05, p<0.01, p<0.001) indicate difference from control. ### (p<0.001) indicates difference from aldosterone alone.

Fig.4. Dose response relationship of mineralocorticoid and glucocorticoid antagonism with the aldosterone effect on Fgf23 transcription. Arithmetic means $\pm$ SEM of Fgf23 mRNA abundance (relative to Tbp mRNA) in UMR106 cells incubated with spironolactone (**A**; n=15), eplerenone (**B**; n=9-11), or mifepristone (**C**; n= 4-6) at the indicated concentration without (white bars) or with (black bars) 100 nM aldosterone (6 h). *, ** (p<0.05, p<0.01) indicate difference from control. #, ### (p<0.05, p<0.001) indicate difference from aldosterone alone.

Figure 1

Figure 2

Figure 3

Figure 4