Ramipril Lowers Plasma FGF-23 in Patients with Diabetic Nephropathy

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Key Words

Endothelial dysfunction · Proteinuria · FGF-23 · ACE inhibitor

Abstract

Background/Aims: Ramipril attenuates renal Fibroblast growth factor-23 (FGF-23) expression, ameliorates proteinuria and normalizes serum phosphate in the diabetic Zucker rat with progressive renal disease suggesting that the reno-protective effect by this drug may be in part due to a FGF-23-lowering effect of angiotensin-converting enzyme (ACE) inhibition. **Methods:** In this nonrandomized study, we tested whether ACE-inhibition reduces circulating FGF-23 in type-2 diabetics with stage-1 chronic kidney disease (CKD) and proteinuria. Intact FGF-23, the eGFR, proteinuria and the endothelium-dependent flow-mediated (FMD) response to ischemia and other parameters were measured at baseline and after 12-weeks of treatment with ramipril (n = 68) or amlodipine (n = 32). **Results:** Blood Pressure (BP) fell to a similar extent (p < 0.001) in the two groups. However, 24 h proteinuria and the FMD improved more (both p < 0.01) in ramipril-treated patients than in amlodipine-treated patients. Changes in proteinuria (r = 0.47) and in FMD (r = -0.49) by ramipril were closely associated (p < 0.001) with simultaneous changes

in FGF-23 and this link was confirmed in multiple regression analyses. In these analyses, the relationship between FMD and proteinuria changes attained statistical significance (p < 0.01) only in a model excluding FGF-23 suggesting that endothelial dysfunction and FGF-23 share a common pathway conducive to renal damage. **Conclusion:** Findings in this study contribute to generate the hypothesis that FGF-23 may be implicated in proteinuria and in endothelial dysfunction in diabetic nephropathy (clinicaltrials.gov (NCT01738945)).

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Introduction

Fibroblast growth factor-23 (FGF-23) is a member of a large superfamily of pleiotropic peptides acting via the FGF receptor (FGFR). FGF-23 is primarily expressed in the skeleton and acts as a circulating hormone endowed with a phosphaturic action [1, 2]. Phosphaturia by FGF-23 requires FGF-23 binding to tubular FGFRs in the presence of a co-receptor, Klotho, which is highly expressed in the kidney [1, 2].

Cohort studies have coherently shown that in patients with chronic kidney disease (CKD) relatively higher se-

rum phosphate levels predict faster CKD progression [3–7]. On the other hand, it is also well established that high levels of the main phosphaturic hormone, FGF-23, predict progression toward kidney failure [8–10], and this finding has been interpreted as a health trade-off to maintain phosphate balance in CKD [11, 12]. Intriguingly, a post-hoc analysis of a clinical trial in proteinuric stage 2–4 CKD patients revealed that high phosphate markedly attenuates the renoprotective effect of ACE inhibitors [7]. A possible explanation of this finding is that ACE inhibition reduces FGF-23 levels in CKD patients, a hypothesis suggested by the experimental observation that ramipril attenuates renal FGF-23 expression along with proteinuria in the diabetic Zucker rat with progressive renal disease [13, 14]. With this background in mind, we explored the hypothesis that ACE-inhibition may reduce circulating FGF-23 in a nonrandomized study, enrolling stage-1 CKD type-2 diabetics with overt proteinuria treated either with ramipril or with amlodipine. Since in previous studies we showed that FGF-23 is associated with endothelial dysfunction and with proteinuria [15], in the present study, we also investigated the relationship between the changes in these outcome measures brought about by ramipril and simultaneous changes in circulating FGF-23 levels.

Materials and Methods

Patients and Controls

The drug ethical committee of Gulhane School of Medicine approved the study and informed consent was obtained by all patients. This retrospective cohort study was registered at clinicaltrials.gov (NCT01738945).

The outpatient clinic of the Department of Nephrology, Gulhane School of Medicine is a large, academic renal care facility, which serves a population of about 2.5 million residents. Our unit has a main interest on endothelial function studies in CKD and we systematically enroll CKD patients in observational studies or drug trials having endothelial function as an outcome measure. In brief, all referred patients who are still untreated are invited to undergo endothelial function studies before starting drug treatment. Furthermore, we apply systematic sampling for biobanking and invite patients to participate in open-label, investigator-initiated, university-funded and industry-independent observational studies or pharmacological trials embedded into clinical practice. As a part of this policy, we had performed two studies in proteinuric patients (>0.5 g/day) with type-2 diabetes and stage-1 CKD (G1A3 category, i.e., normal to high GFR and severely increased albuminuria) [16], one testing the effect of an ACE inhibitor (ramipril) on inflammation biomarkers and endothelial function [17] and another investigating the influence of a calcium blocker (amlodipine) on the same outcome measures [18]. These studies had exactly the same duration (12 weeks) and adopted identical inclusion criteria, i.e., in order to be enrolled, patients had to be (a) type-2 diabetes, (b) older than 18 years, (c) non-smoker, (d) cardiovascular events

free, (e) non-obese ($\text{BMI} < 30 \text{ kg/m}^2$), (f) with daily protein excretion $>500 \text{ mg/day}$, (g) with systolic blood pressure $\geq 140 \text{ mm Hg}$ and/or diastolic blood pressure $\geq 90 \text{ mm Hg}$, (h) with serum cholesterol $<200 \text{ mg/dl}$ and never treated with statins, (i) with no previous treatment with ACE inhibitors or angiotensin II blockers.

Forty-nine of the patients who had participated into the ramipril study [17] plus 29 patients recruited subsequently formed the ramipril cohort of the present study. Eleven of these twenty-nine patients dropped out during the first three weeks of ramipril treatment because of cough ($n = 3$), hyperkalemia ($n = 2$), or for logistic reasons or because they were non-compliant with ramipril treatment ($n = 6$). Overall, 67 patients of the ramipril arm completed this study.

The amlodipine arm was formed by the 32 out of 35 type-2 diabetics with available plasma samples who participated into the previously mentioned amlodipine study [18].

Intervention and Follow-Up Measurements

After baseline measurements of renal function parameters (eGFR, proteinuria), vascular function studies and blood sampling for the measurement of FGF-23 (see below), patients were treated either with an ACE inhibitor (ramipril, 5 mg/day) or with a calcium channel blocker (amlodipine, 10 mg/day) for 12-weeks at which time the same measurements were repeated. During the study period, serum creatinine and potassium concentrations were measured every 2 weeks and the dose of ramipril was titrated to achieve maximum reduction of 24 h proteinuria without significant hypotensive episodes and/or a serum potassium concentration rising $>5.5 \text{ mEq/l}$. As described above, 11 patients in the ramipril arm were excluded because of ACEI treatment side effects, or due to noncompliance. Thus, 67 patients were included in the final analysis. None of the patients with amlodipine group ($n = 32$) dropped out during the 12-week study period.

Blood Chemistry

Morning blood samples were collected from patients and control subjects after 12 hours of fasting was done at baseline and after 12 weeks, before the endothelial function studies. Subjects were asked to refrain from physical activity for at least 30 minutes prior to the blood draw. Measurements of total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) cholesterol and fasting plasma glucose (FPG) were obtained in the routine clinical laboratory using an Olympus AU 600 auto analyzer and reagents from Olympus Diagnostics GmbH (Hamburg, Germany). C Reactive protein, Low-density lipoprotein (LDL) cholesterol, serum total calcium and phosphorus were described in detail in previous papers [15–17]. All samples were run in triplicates. The glomerular filtration rate (GFR) was calculated according to the simplified version of the Modification of Diet in Renal Disease (MDRD) Study prediction equation formula [$\text{GFR} = 186 \times \text{Pcr}^{-1.154} \times \text{age}^{-0.203} \times 1.212$ (if black) $\times 0.742$ (if female)] [19].

Serum FGF-23 and Biomarkers of Mineral Metabolism

Intact FGF-23 was measured using an ELISA according to the manufacturer's protocol (Kainos Laboratories International; Tokyo, Japan). This second-generation, two-site, monoclonal antibody ELISA has previously been shown to recognize the biologically active, intact FGF-23. The Kainos Intact FGF-23 assay has a lower limit of detection of 3 pg/ml and intraassay and interassay coefficients of variation of less than 5%. The calculated overall intra-assay coefficient of variation was 2.5%, and the calculated

Table 1. Clinical and biochemical data before and after the pharmacological intervention with ramipril and amlodipine in the two study arms

	Baseline (before ramipril) (n = 67)	Follow-up (12 weeks of ramipril) (n = 67)	p*	Baseline (before amlodipine) (n = 32)	Follow-up (12 weeks of amlodipine) (n = 32)	p**
Age, years	48±7			47±5		
Gender, M/F	33/34			14/18		
BMI, kg/m ²	26.5±1.9	26.4±2.5	0.13	26.5±2.1	25.5±2.1	0.06
Total cholesterol, mg/dl	177.7±34.9	175.9±30.7	0.68	178.7±23.5	174.9±29.2	0.61
Triglycerides, mg/dl	151.5±33.8	156.7±32.9	0.26	152.5±21.4	154.1±34.1	0.79
LDL cholesterol, mg/dl	116.7±30.3	113.1±27.4	0.43	119.7±27.5	118.1±37.1	0.79
Glucose, mg/dl	168.9±21.4	142.3±20.7	0.01	160.5±22.9	154.8±23.2	0.30
SBP, mm Hg	140±9	129±9	<0.001	139±8	129±7	<0.001
DBP, mm Hg	87 (82–90)	80 (80–87)	<0.001	88 (85–90)	80 (80–85)	<0.001
eGFR, ml/min/1.73m ²	113 (105–119)	106 (92–116)	<0.001	119 (110–122)	106.5 (97–116)	<0.001
Serum albumin, g/dl	3.9±0.4	4.0±0.3	0.31	3.8±0.3	3.9±0.3	0.55
24 h proteinuria, mg/day	1,590 (1,300–1,780)	700 (530–1,110)	<0.001	1,625 (1,302–2,070)	1,200 (990–1,618)	0.003
hs-CRP, mg/l	12 (5–24)	8 (2–15)	<0.001	11.5 (8–17)	8.5 (5–11)	0.003
Serum calcium, mg/dl	9.1±0.5	9.1±0.5	0.32	9.2±0.5	9.0±0.5	0.19
Serum phosphate, mg/dl	3.9±0.6	4.0±0.6	0.84	4.1±0.5	4.1±0.4	0.30
iPTH, pg/ml	54 (41–66)	52 (41–65)	0.35	49 (39–61)	55 (45–66)	0.12
25OH vitamin D, nmol/l	52.4±11.4	51.3±13.9	0.56	48.6±11.5	44.9±10.7	0.10
FGF-23, pg/ml	40.5 (26–80)	28.0 (22.5–34.3)	<0.001	40.5 (30.9–50.9)	40.7 (31.5–51.0)	0.96
NMD, %	12.9±0.4	13.1±0.5	0.07	13.0±0.4	13.1±0.5	0.19
FMD, %	6.6 (6.1–7.1)	8.0 (7.4–8.6)	<0.001	6.8 (6.3–7.0)	7.0 (7.0–7.9)	0.002

BMI = Body mass index; SBP and DBP = systolic and diastolic BP; FMD = flow-mediated vasodilatation; NMD = nitroglycerin-mediated dilatation. Mean ± SD, median (interquartile range).

* Paired samples t test comparing baseline and 12-week values in the ramipril group.

** Paired samples t test comparing baseline and 12-week values in the amlodipine group.

overall inter-assay coefficient of variation was 2.8%. We measured all samples in duplicate.

To measure 25OHVD, we used high-performance liquid chromatography (HPLC) kits following the manufacturer's instructions (ImmuChrom GmbH, Heppenheim, Germany). Quantification of 25-OH vitamin D3 was made by the HPLC system with UV (264 nm) detector (Thermo Electron, San Jose, Calif., USA). The intra-assay coefficient of variation was 0.9–2.9%, and the calculated inter-assay coefficient of variation was 1.7–3.9% and recovery was 91%. Intact parathormone was measured by IRMA, using a commercial kit (Immolute Intact PTH) from Diagnostic Product Corporation (Los Angeles, CA) with a sensitivity of 1 pg/ml.

Endothelial Function Tests

All vascular function studies were performed in the morning. Arterial pressure was measured three times after a 15-min resting period and mean values were calculated for systolic and diastolic pressures for all patients. Endothelium-dependent flow-mediated vasodilatation (FMD) and endothelium-independent vasodilatation (nitroglycerin-mediated dilatation [NMD]) of the brachial artery was assessed noninvasively, using high-resolution ultrasound as described by Celermajer et al. [20]. The method for the vascular assessment in our laboratory met the criteria, which were mentioned by the International Brachial Artery Reactivity Task Force

[21] and was described in full detail in previous publications. All ultrasound images were recorded on S-VHS videotape for subsequent blinded analysis. The maximum FMD and NMD diameters were calculated as the average of the three consecutive maximum diameter measurements after hyperemia and nitroglycerin, respectively. The FMD and NMD levels were then calculated as the percent change in diameter compared with baseline resting diameters.

Statistical Analysis

All analyses were performed by using the SPSS 15.0 (SPSS Inc., Chicago, Ill., USA) statistical package. Non-normally distributed data are expressed as median (range) and normally distributed data as mean ± SD as appropriate. A p value <0.05 was considered statistically significant. One sample Kolmogorov Smirnov test was used for the analysis of data distribution. One way ANOVA, the student *t* test and the paired sample *t* test were used for comparing unpaired and paired data. Standard correlation analysis was applied to determine the associations between continuous variables. Finally, multiple regression analysis was used to test the independent link between changes in proteinuria (outcome variable) and FGF-23 levels in response to ramipril treatment. In this analysis, we included variables that underwent significant (*p* < 0.05) changes after ramipril treatment (vs. baseline, see table 1).

Results

At baseline (table 1), the ramipril and the amlodipine groups were similar for age, gender, serum lipids, BP, eGFR, proteinuria, serum glucose, albumin, CRP and biomarkers of chronic kidney disease-bone mineral disorder (CKD-BMD) disorder (PTH, serum Ca and P, and FGF-23). After the 12-week drug treatment intervention, BP fell to a similar extent ($p < 0.001$) in the two groups (ramipril -11 ± 12 mm Hg/ -5 ± 5 mm Hg; amlodipine -11 ± 7 mm Hg/ -7 ± 6 mm Hg) and the BP-lowering effect was accompanied by a similar fall in the eGFR (ramipril: -7 ± 14 ml·min $^{-1}$ ·1.73 m ^{2} ; amlodipine: -10 ± 12 ml·min $^{-1}$ ·1.73 m ^{2} , $p < 0.001$) value. However, 24 h proteinuria showed a more marked reduction in ramipril-treated patients (-834 ± 545 mg/24 h) than in amlodipine-treated patients (-438 ± 773 mg/24 h) ($p < 0.001$).

Serum glucose fell by 27 mg/dl in the ramipril group ($p < 0.01$) and by 6 mg/dl ($p = 0.30$) in the amlodipine group. Serum Ca, P, PTH, 25OH vitamin D did not change in either group.

As shown in figure 1 and table 1, serum FGF-23 fell on average by the $31.51 \pm 23.82\%$ from a median value of 40 pg/ml (interquartile range: 26–80 pg/ml) to 28 pg/ml (18–55 pg/ml) in the ramipril group ($p < 0.001$) but remained unchanged [40 pg/ml (28–64 pg/ml) vs. 41 (21–65 pg/ml)] in the amlodipine group and the difference in FGF-23 levels between the groups was highly significant ($p < 0.001$). Flow-mediated vasodilatation increased in both groups, but the magnitude of this phenomenon was more marked ($p \leq 0.001$) in the ramipril ($20 \pm 14\%$) than in the amlodipine ($8 \pm 13\%$) group (fig. 1). Changes in FGF-23 in ramipril-treated patients went along with simultaneous changes in FMD and in proteinuria (fig. 2 and table 2) but were independent of changes in BP, eGFR and other variables, including serum phosphate, which was unaltered by ramipril treatment (table 1). No relationship between proteinuria and FGF-23 changes was registered in the amlodipine group. No significant change in nitroglycerin-mediated vasodilatation was observed in either group [ramipril: baseline: $12.9 \pm 0.4\%$, 12th week: $13.1 \pm 0.5\%$ ($p = 0.07$); amlodipine $13.0 \pm 0.4\%$, 12th week: $13.1 \pm 0.5\%$ ($p = 0.19$)].

Ramipril Effects on Proteinuria and FMD: Multiple Regression Analyses

Because the decline in proteinuria induced by ramipril was more marked than that induced by amlodipine and was strictly related to simultaneous changes in FMD and in FGF-23, we performed multiple regression analyses to

Fig. 1. FGF-23, proteinuria and flow mediated vasodilatation (FMD before and after ramipril or amlodipine).

assess the independent correlates of changes in proteinuria by this drug. In this analysis, we included the variables that changed (12 week-baseline) to a significant extent ($p < 0.05$) during ramipril treatment, namely BP, glucose, eGFR, CRP, FGF-23 and FMD. As shown in table 2, in this analysis FGF-23 emerged as the sole significant covariate explaining the variability in changes in proteinuria. However, in a model with the same variables but excluding FGF-23, FMD as a strong independent correlate of proteinuria ($\beta = -0.31$, $p = 0.01$) suggested that FGF-23 and FMD are in the same pathway conducive to proteinuria.

Discussion

In this study, we found that ACE inhibition by ramipril, but not calcium channel antagonism by amlodipine, lowers plasma FGF-23 levels in stage 1-CKD type-2 diabetic

Fig. 2. Relationship between FGF-23 changes brought about by ramipril and changes in proteinuria and in FMD brought about by the same drug.

Table 2. Correlates of changes in proteinuria following ACEI therapy: unadjusted and adjusted analyses

	Changes in proteinuria (mg/day) induced by ramipril	
	univariate beta (p)	multivariate beta (p)
Changes in FGF-23, %	0.64 (<0.001)	0.64 (<0.001)
Changes in FMD, %	-0.31 (0.01)	0.03 (0.83)
Changes in SBP, mm Hg	-0.08 (0.52)	-0.14 (0.27)
Changes in DBP, mm Hg	-0.13 (0.28)	-0.09 (0.47)
Changes in serum glucose, mg/dl	-0.19 (0.12)	0.01 (0.98)
Changes in hsCRP, mg/l	0.10 (0.41)	0.04 (0.78)
Changes in eGFR, mg/dl	-0.22 (0.08)	0.07 (0.57)

patients with proteinuria. Furthermore, the anti-proteinuric effect of ramipril was strongly associated with simultaneous changes in FGF-23 levels, while no such relationship was found in patients treated with amlodipine. Overall, these findings suggest that FGF-23 is implicated in proteinuria in diabetic patients with normal GFR and that the favorable effect of ACE inhibition may in part depend on the FGF-23-lowering effect of this intervention.

Seminal observations in the remnant kidney model by Ibels [22] in the late seventies showed that phosphate excess promotes calcium phosphate precipitation in the renal interstitium, severe renal damage and progression to

uremia in this model. More recent studies documented that high phosphate activates additional pathways that may be conducive to renal damage, that is, oxidative stress and endothelial dysfunction [23]. Due to the noxious effects of high-burden phosphate on the kidney by these pathways, it was hypothesized that progression to kidney failure may be a trade-off for the maintenance of phosphate balance in the face of reduced nephron mass in CKD patients [11]. The augmented phosphate excretion per functioning nephron depends on complex adaptations in the hormonal system, which regulates mineral metabolism, and fibroblast growth factor 23 (FGF-23) has a prominent role in these adaptations [2, 11, 24, 25]. Mechanism(s) whereby FGF-23 may cause renal damage include cell proliferation [26] inhibition of the synthesis of 1,25 OH₂ vitamin D and activation of the renin-angiotensin-system (RAS) [27, 28]. In the diabetic Zucker rat with progressive renal disease, ACE inhibition by ramipril reduces the expression of FGF-23 in the kidney and attenuates proteinuria [13]. Furthermore, FGF-23-mediated inhibitory effects of phosphate on nitric oxide (NO) production might also contribute to renal damage via endothelial dysfunction [15], a risk factor for renal disease in experimental models and an established regulator of local angiotensin II in the kidney. Cognate to these observations, are findings in experimental models showing that the co-receptor of FGF-23, Klotho, whose plasma levels mirror those of FGF-23 in CKD patients [29], is a renoprotective factor [30, 31], mitigating kidney damage

by angiotensin II [32] and a fundamental factor regulating the expression of nitric oxide synthase and the attendant endothelial function in mice [33]. The potential relevance of RAS in renal damage by high FGF-23 in humans is also suggested by the findings in a post-hoc analysis of the REIN study showing that high phosphate, the main factor regulating circulating FGF-23 levels, markedly attenuates the renoprotective effect of ramipril in patients with proteinuric nephropathies [7]. However, FGF-23 levels were not measured in the REIN study.

In the present study, ramipril produced a 30% decline in FGF-23 levels. Such a change appeared peculiar to ACE inhibition because treatment with a calcium antagonist like amlodipine failed to modify FGF-23 levels. As expected, ramipril also produced an important decline in proteinuria (–56% on average) and changes in proteinuria by this drug went along with the simultaneous change in FGF-23, suggesting that this growth factor may have a role in renal damage. Furthermore, in line with previous observations in stage 4-CKD showing an inverse relationship between changes in serum FGF-23 brought about by phosphate-lowering treatments and changes in the endothelium-dependent flow-mediated vasodilatation [34], we found that ramipril-induced changes in FGF-23 paralleled those in endothelial function further supporting the hypothesis that FGF-23 plays a role in endothelial dysfunction in CKD. Observations in the present study are in keeping with the recent findings in the diabetic Zucker rat with reduced GFR where ramipril suppresses the renal expression of FGF-23 also attenuating proteinuria and renal injury [14]. Overall, these data generate the hypothesis that the effect of ACE-inhibitors, the class of drugs endowed with the most pronounced anti-proteinuric and nephroprotective effect in clinical studies, is at least in part mediated by their FGF-23 lowering ability.

Due to nonrandomized design, this study has inherent limitations that should be clearly acknowledged. First, although the demographic, clinical and biochemical pa-

rameters of the ramipril and the amlodipine arm of the study was similar, the possibility that unmeasured risk factors might have confounded the apparently different effect of ramipril and amlodipine on FGF-23 and other variables cannot be excluded. Second, we pragmatically focused on type-2 diabetics with proteinuria with normal GFR because we could access a bio-bank connected to the previous trial performed at our institution, and therefore, the findings in this study cannot be extrapolated to other nephropathies and other CKD stages. Another limitation is the lack of data on dietary phosphate/calcium intake and phosphaturia. Furthermore, we did not measure Klotho, a fundamental FGF-23 co-receptor closely related with angiotensin II-related renal damage [32].

In conclusion, this study generates the hypothesis that nephroprotection by ACE inhibition may in part depend on the fact that this intervention lowers plasma FGF-23 levels. Such a hypothesis is supported by the strong association between the FGF-23-lowering effect of ramipril with the anti-proteinuric and endothelium-protective effect of this drug; such a hypothesis remains to be tested in detailed mechanistic studies in experimental models and in appropriate randomized clinical trials in CKD patients.

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Disclosure Statement

The authors have no relationship or financial interests with companies related to the findings of this work.

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