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A protective role for FGF-23 in local defence against disrupted arterial wall integrity?

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ABSTRACT

Increasing interest is focusing on the role of the FGF-23/Klotho axis in mediating vascular calcification. However, the underpinning mechanisms have yet to be fully elucidated. Murine VSMCs were cultured in calcifying medium for a 21 d period. FGF-23 mRNA expression was significantly up-regulated by 7 d (1.63-fold; $P < 0.001$), with a concomitant increase in protein expression. mRNA and protein expression of both FGFR1 and Klotho were confirmed. Increased FGF-23 and Klotho protein expression was also observed in the calcified media of *Enpp1*$^{-/-}$ mouse aortic tissue. Reduced calcium deposition was observed in calcifying VSMCs cultured with recombinant FGF-23 (10 ng/ml; 28.1% decrease; $P < 0.01$). Calcifying VSMCs treated with PD173074, an inhibitor of FGFR1 and FGFR3, showed significantly increased calcification (50 nM; 87.8% increase; $P < 0.001$). FGF-23 exposure induced phosphorylation of ERK1/2. Treatment with FGF-23 in combination with PD98059, an ERK1/2 inhibitor, significantly increased VSMC calcification (10 μM; 41.3% increase; $P < 0.01$). Use of FGF-23 may represent a novel therapeutic strategy for inhibiting vascular calcification.

1. Introduction

Vascular calcification is a marker of increased cardiovascular risk in ageing, and in a number of diseases including diabetes, atherosclerosis and chronic kidney disease (CKD) (Demer and Tintut, 2008; Mackenzie and MacRae, 2011; Zhu et al., 2012). Although condition-specific factors are likely to drive the calcification process, the etiology of mineral accumulation within the vasculature shares many similarities with that of bone formation (Demer and Tintut, 2008; Shroff and Shanahan, 2007). Indeed, a number of studies have reported that vascular smooth muscle cells (VSMCs), the predominant cell type involved in vascular calcification, can undergo phenotypic transition to osteoblastic, chondrocytic and osteocytic cells in a calcified environment (Speer et al., 2005; Zhu et al., 2011). Furthermore, it has been demonstrated that phosphate accelerates this phenotypic trans-differentiation, evident in the loss of characteristic smooth muscle markers and the development of osteoblastic features, such as the expression of tissue-nonspecific alkaline phosphatase, P_iT-1, osteocalcin and osteopontin, and osteocyte markers including sclerostin and E11 (Speer et al., 2009; Zhu et al., 2011). Vascular calcification also involves the reciprocal loss of recognised calcification suppressors, such as inorganic pyrophosphate (PP_i), MGP and fetuin A (Murshed et al., 2005; Rutsch et al., 2003).

The family of Fibroblast Growth Factors (FGFs) consists of 23 proteins that regulate cell proliferation, migration, differentiation and survival (Eswarakumar et al., 2005). FGF-23, the most recently discovered FGF, is produced by osteocytes in bone and regulates phosphate homeostasis via signalling through its receptors (mainly FGFR1) in the presence of Klotho, its cofactor in the kidney and parathyroid glands (Kurosu et al., 2006; Shimada et al., 2001; Urakawa et al., 2006). The primary physiological actions of FGF-23 are to augment phosphaturia by downregulating the expression of type IIa and IIc sodium-phosphate transporters within the renal proximal tubular cells and to decrease circulating concentrations of 1,25-dihydroxyvitamin D_3 via inhibition of 1α hydroxylase activity (Kurosu et al., 2006; Wolf, 2010). FGF-23 also negatively regulates parathyroid hormone (PTH) secretion (Ben-Dov et al., 2007).

Increasing interest is focusing on the role of the FGF-23/Klotho axis in mediating vascular calcification. A direct correlation between FGF-23 circulating levels and the extent of aortic calcium deposition in mice fed a high-phosphate diet has been recently demostrated (El-Abbadi et al., 2009). An association between FGF-23 levels and calcium accumulation in the arteries of dialysis patients has also been reported (Srivaths et al., 2011). Increased circulating FGF-23 levels have also been observed in the *Enpp1*$^{-/-}$ mouse model of medial vascular calcification (Mackenzie et al., 2012a), as well as in patients with hypophosphatemic rickets resulting from a loss of function mutation in the *ENPP1* gene (Lorenz-Depiereux et al., 2010). In patients with CKD, increased

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FGF-23 plasma levels have been linked to a decrease in kidney function, the presence of vascular damage and an increased risk of cardiovascular mortality (Isakova et al., 2011; Nasrallah et al., 2010; Srivaths et al., 2011; Yilmaz et al., 2010). However, both clinical and basic studies have demonstrated conflicting evidence as to whether FGF-23 imparts a protective or a harmful role on the vasculature during stress. FGF-23 may therefore maintain vascular health at physiological levels, and may only at high circulating concentrations exert harmful effects. Interestingly, recent studies have suggested that FGF-23 directly inhibits vascular calcification (Lim et al., 2012; Razzaque and Lanske, 2007; Shalhoub et al., 2012). However it has also been suggested that elevated FGF-23 concentrations may stimulate vascular calcification by acting directly on the vascular wall to induce a local reduction of Klotho (Donate-Correa et al., in press). Therefore, in the present study, we have undertaken *in vitro* and *ex vivo* murine VSMC calcification studies to provide fundamental insights into the expression profiles of FGF-23 during vascular calcification. Further investigations have provided novel insights into the functional role and underpinning mechanisms of FGF-23 in protecting VSMCs from pathological calcification.

2. Materials and methods

2.1. Enpp1$^{-/-}$ mice

Enpp1$^{-/-}$ mice were generated and characterised as previously described (Sali et al., 1999). Genotyping was performed by a commercial genotyping service (Genetyper, New York, USA) using genomic DNA isolated from ear clips. All animal experiments were approved by The Roslin Institute's Animal Users Committee and the animals were maintained in accordance with Home Office guidelines for the care and use of laboratory animals.

2.2. Primary murine VSMC isolation

Primary VSMCs were isolated from 5-week old wild-type (WT) C57BL/6 mice as previously described (Johnson et al., 2008). Briefly, after removal of adventitia, the aorta was cut open to expose the endothelial layer. Tissues from eight animals were pooled for digestion with 1 mg/ml trypsin for 10 min in order to remove any remaining adventitia and endothelium. Following a further overnight incubation at 37 °C in a humidified atmosphere of 95% air/5% CO_2 in growth medium consisting of α-MEM (Invitrogen, Paisley, UK) supplemented with 10% FBS (Invitrogen) and 1% gentamicin (Invitrogen), tissues were then digested with 425 U/ml collagenase type II (Worthington Biochemical Corporation, Lakewood, USA) for 5 h. Isolated VSMCs were expanded in growth medium for two passages in T25 tissue culture flasks (Greiner Bio-one, GmbH, Frickenhausen, Baden-Wurttemberg, Germany) coated with 0.25 μg/cm^2 murine laminin (Sigma, Poole, UK) to promote maintenance of the contractile differentiation state (Johnson et al., 2008).

2.3. Cell culture

Primary VSMCs were seeded in growth medium at a density of 1.5×10^4/cm^2 in multi-well plates. At confluency (day 0), VSMCs were cultured in growth medium supplemented with 2.5 mM β-glycerophosphate (βGP) (Sigma) and 50 μg/ml ascorbic acid (AA) (Sigma) or 3 mM Na_2HPO_4/NaH_2PO_4 (P_i) (Sigma) for up to 21 d to induce calcification. Cells were maintained in 95% air/5% CO_2 and the medium was changed every third/fourth day.

Recombinant mouse FGF-23 (R&D Systems, Abingdon, UK) at 10–50 ng/ml was added to cultures at confluence for up to 9 days. PD98059 (Sigma) at 10 μM and PD173074 (Source Bioscience, Nottingham, UK) at 10 and 50 nM were also added at confluence in 0.1% DMSO to inhibit Erk1/2 signalling and FGFR1, respectively. Control cultures received 0.1% DMSO only. Cell viability was assessed using a commercially available kit (Alamar Blue; Invitrogen).

2.4. Detection of calcification

Calcium deposition was evaluated by staining the cell-matrix monolayer with alizarin red (Sigma) as previous described (MacRae et al., 2010). In brief, VSMCs were washed twice with phosphate buffered saline (PBS), fixed in ice-cooled 4% paraformaldehyde (PFA) for 5 min at 4 °C, stained with 2% alizarin red (pH 4.2) for 10 min at room temperature and rinsed with distilled water. Alizarin red stained cultures were extracted with 10% cetylpyridium chloride for 10 min and the O.D. was determined at 570 nm by spectrophotometery (Multiskan Ascent, Thermo Electron Corporation, Vantaa, Finland). Calcium deposition in VSMCs was also assessed by HCl leaching. Cells were decalcified in 0.6N HCl overnight and free calcium determined colorimetrically by a stable interaction with phenolsulphonethalein using a commercially available kit (Randox Laboratories Ltd., County Antrim, UK) and corrected for total protein concentration, following extraction using 1 mM NaOH in 0.1% SDS (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK).

2.5. Alkaline phosphatase (ALP) activity

Cell layers were lysed with 0.9% NaCl and 0.2% Triton X-100 and centrifuged at 12 000 g for 15 min at 4 °C. The supernatant was assayed for protein content and ALP activity. Enzyme activity was determined by measuring the cleavage of 10 mM p-nitrophenyl phosphate (pNPP) at 410 nm using a commercially available kit (Thermo Trace, Melbourne, Australia). Total ALP activity was expressed as nmoles pNPP hydrolysed/min/mg protein (Mackenzie et al., 2011; Zhu et al., 2011).

2.6. Real-time-polymerase chain reaction (RT-PCR)

Total RNA was isolated from VSMCs using RNeasy total RNA (Qiagen Ltd., Crawley, West Sussex, UK), according to manufacturer's instructions. RNA was quantified and reversed transcribed as previously described (MacRae et al., 2006a, 2009). All genes were analysed with the SYBR green detection method (Roche, East Sussex, UK) using the Stratagene Mx3000P real-time QPCR system (Stratagene, CA, USA). Each PCR was run in triplicate. All gene expression data were normalised against *Gapdh* and the control values expressed as 1 to indicate a precise fold change value for each gene of interest. Primers for *Runx2* forward 5'-ACC ATA ACA GTC TTC ACA AAT CCT-3 and reverse 5′CAG GCG ATC AGA GAA CAA ACT A-3, *Pit-1* forward 5′-CAC TCA TGT CCA TCT CAG ACT-3 and reverse 5′-CGT GCC AAA GAA GGT GAA C-3, *Fgf-23* forward 5′-GGA TCT CCA CGG CAA CAT TT-3 and reverse 5′-GTA GTG ATG CTT CTG CGA CAA-3, *Osteocalcin* (*Ocn*), tissue non-specific alkaline phosphatase (*Alpl*), *Klotho, FgfR1* and *FgfR3* (Qiagen; sequence not disclosed) and *Gapdh* (Primer Design, Southampton, UK; sequence not disclosed) were used.

2.7. Western blotting

Cultured cells were lysed in RIPA buffer (Invitrogen) containing "complete" protease inhibitor cocktail according to manufacturer's instructions (Roche). Immunoblotting was undertaken as previously described (MacRae et al., 2006b, 2009). Recombinant mouse FGF-23 and Klotho were used as positive controls (R&D Systems).