Role of FGF23 signalling in aortic nitric oxide production and bioavailability

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1. Introduction

1.1. Physiology of Vitamin D₃ (1α,25-dihydroxyvitamin D₃) activation

Provitamin D (7-Dehydrocholesterol) is unique upon the series of vitamins in mammals. It is stored in the epidermis of the skin and following the sunlight UVB radiation exposure could be converted to the precursor substances of the biological active form. This mechanism evolved nearly 500 million years ago when sunlight was discovered to be an energy source for the organisms. Diverse photochemical reactions found to be important for the essential energy metabolism processes starting from the lower eukaryotes like phytoplankon (*Emiliania huxleyi*) (Wacker and Holick 2013), plants and fungi, and going down to the mammals during evolutionary development. Since physiological functions of provitamin D active form 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) was described as an essential for the mineral homeostasis regulation in the vertebrates the mechanistical regulation and the physiological aspects of so called vitamin D endocrine system since many years subjected as a research focus constantly exploding basic science and also clinically relevant knowledge in this area (Bouillon and Suda 2014).

The molecular mechanism for 1α,25-dihydroxyvitamin D₃ production involved vitamin D binding protein (DBP) transporting transvitamin D₃ to the liver, where hydroxylated 25(OH)-hydroxyvitamin D₃ is produced. Final activation of the Vitamin D₃ occurs in the proximal tubules cells of the kidney as an additional hydroxyl-group is added on the 1α position of the sterol derivate. The activated vitamin D₃ is then secreted in the circulation and transported to the divorce target organs in the body. The enzyme required for vitamin D₃ activation is 1-α hydroxylase (1α-OHase) known to be mainly expressed in the kidney (D Zehnder et al. 1999). However in the last years an extra-renal function of 1α-OHase was described. For instance expression was detected in the macrophages and endothelial cells (Stoffels et al. 2007) participating to the so called local tissue specific vitamin D production system, which is believed to be even more ancient in phylogenetic history (Adams and Hewison 2012).

In humans only approximately 10 – 20% of the total circulating vitamin D originates from dietary sources (Dalan et al. 2014). Therefore, sunlight UVB radiation is essential for a
normal vitamin D homeostasis in humans. It is reported that the skin pigmentation type is strongly correlated with light penetration into the epidermis and therefore the with the epidermal vitamin D precursor conversion. (Clemens et al. 1982). Also the zenith angle, seasoning and ecological factors also effect vitamin D production physiology and has to be considered for the vitamin D supplementation strategy in the clinic. It is well known that in winter months, limited sun light exposure leads to the vitamin D deficiency in Northern living population (Webb et al. 1988) and requiring more precise vitamin D monitoring in these regions.

Vitamin D acts through its specific nuclear receptor (VDR) a ligand-activated transcription factor that function to control gene expression. The two DNA half-sites accommodate the binding of a heterodimer comprised of a VDR molecule and a retinoid X receptor (RXR) molecule. Binding of the to a VDR-response element to the genomic DNA is regulated by co-activating proteins, which are determining tissue specific action of the vitamin D (Issa et al. 2010). The final complex in the VDR transcriptional regulation requires recruitment of the RNA-polymerase II to start transcription of vitamin D associated genes (Morris 2014).

Although a number of rapid and non-genomic actions of vitamin D have been described the vast majority of the effects of the hormone are mediated by the VDR, which is the only protein that binds vitamin D effectively at sub-nanomolar concentrations.

In parallel to the canonical molecular signalling of vitamin D recent findings suggest a steroid membrane-associated receptor (MARRS) as an additional vitamin D driven signalling activator (Khanal and Nemere 2007). This rather rapid intracellular response to the vitamin D is involved in the cancer growth impairment as well as (Richard et al. 2010) contribute to the regulation of the intestinal minerals uptake (Nemere et al. 2012).

1.2. Role of vitamin D in calcium and phosphate homeostasis regulation

The main physiological actions of the vitamin D are calcium and phosphorus uptake and transport regulation and thereby controlling of the bone formation. When systemic concentrations of the calcium and/or phosphate drops, the vitamin D endocrine system activates calcium and phosphate retrieval through enhancing reabsorption in the small intestine and in the kidney distal tubules cells (Morris 2014). At the same time action of the
vitamin D on the skeleton triggers osteoclast-mediated bone resorption activating mobilization of the calcium and phosphate stores to compensate circulating levels. All three renal, intestinal and skeletal actions of the VDR signalling are directed to support the balance of calcium and phosphate levels in the blood.

In enterocytes of the small intestine the formed 1,25(OH)₂D₃-VDR-RXR complex is able to bind its intrinsic response element on the DNA that promote transcription of: (1) calcium specific ion channels (TRPV5/6), (2) intracellular transport proteins for calcium (e.g. calbindin D9k) or (3) the membrane calcium ATPase (PMCA) (Christakos et al. 2003). A similar system is described for phosphate (Kido et al. 2013).

Similar regulatory mechanisms were also found in the kidney, where vitamin D activates calcium reabsorption of the distal tubules, via calcium channels TRPV5 and intracellular transport calcium proteins calbindin D9k and calbindin 28k resulting in the elevation of the calcium blood level (Haussler et al. 2008).

In the bone, VDR-depended calcium and phosphate homeostasis regulation stimulate osteoclasts production of NFκB-ligand RANKL, which lead to an enhanced maturation of these cells (Pike 2011). This stimulates bone resorption and finally increased systemic available calcium and phosphate levels increases.

1.3. Extra-renal activation and non-classical functions of Vitamin D

Even though these classical effects of vitamin D provide a complex field on its own, there are other relevant implications of this cholesterol derivate. The enzyme 1α-hydroxylase (Cyp27b1), which is catalysing the activation of 1,25(OH)₂D₃ shows expression in an variety of tissues, including pancreas, lymph nodes, brain, placenta or macrophages (D Zehnder et al. 2001). In contrary to the kidney the activation in extra-renal sites does not contribute to the systemic vitamin D level but rather has some local effects, such as cell proliferation, differentiation, or anti-apoptotic signalling (Höbaus et al. 2013). Studies reveal that even in malignancy the local activation of 1,25(OH)₂D₃ via 1α-hydroxylase may prevent tumor growth, due to its antimitotic effects. Interestingly, in the early stage of the tumor development the cells tend to overexpress the enzyme, whereas lose their ability to produce 1,25(OH)₂D₃ in an later phase (Bises et al. 2004).
In extra-renal tissue, which can produce active vitamin D, the expression is on the one hand mainly regulated via the substrate and its bioavailability. Therefore vitamin D systemic levels modulated by dietary and the environmental factors can regulate peripheral sites of the tissue specific vitamin D sources (Daniel Zehnder et al. 2002a). On the other hand there is evidence that extra-renal 1α-OHase is also transcriptionally down-regulated by FGF23 with an extracellular-signal regulated kinase (Erk1/2) pathway likewise in proximal tubules cells (Chanakul et al. 2013). Studies reveal that the endothelium provides a microendocrine vitamin D system, containing the VDR and 1α-OHase, which maintains vascular homeostasis in an autocrine/paracrine manner (Merke et al. 1989). Vitamin D₃ can prevent oxidative stress dependent down regulation of the VDR and antioxidative CuZn-superoxide dismutase (SOD) in endothelial cells (Zhong et al. 2014). Thus local produced vitamin D could be involved in anti-oxidative signalling, as shown through an Erk 1/2 mediated pathway (Polidoro et al. 2013).

1.4. Vitamin D regulates endothelial NO production

Nitric oxide (NO) is one of the most important vasodilators produced by the endothelium itself. The protein endothelial nitric oxide – synthase (eNOS) can cleave NO from the amino acid L-arginine and release it as a gasotransmitter, which diffuses freely into vascular smooth muscle cells and initiate a loss of calcium sensibility (Akata 2007). This ultimately leads to a decrease in the vascular tone through activation of the vascular smooth muscle cells relaxation. Loss or dysfunction of this system is therefore likely to cause cardiovascular pathologies (Davel et al. 2011).

Human epidemiological data provide that vitamin D deficiency is a key parameter for developing a series of cardiovascular diseases, such as hypertension or endothelial dysfunction (Al Mheid et al. 2011). Increased exposure to UVB radiation was positively associated with a lower blood pressure in hypertension patients (Krause et al. 1998). However beneficial effect of vitamin D supplementation on the cardiovascular outcomes still remains controversial (Forman et al. 2013). Recent studies of our group have shown that vitamin D is a direct transcriptional regulator of the eNOS. The functional loss of the vitamin D receptor (VDR), increased significantly the arterial stiffness and cardiac afterload in 3 month old mice.
Animals lacking the gene for eNOS (Nos3) develop a similar phenotype, indicating that vitamin D is a key regulator of Nos3-activation (Andrukhova et al. 2014).

**1.5. Vitamin D and FGF23/Klotho signalling interaction in phosphate homeostasis regulation**

Phosphate is involved in a variety of essential cellular processes, such as protein signalling, energetic metabolism or nucleic acid synthesis, maintenance of the physiological pH etc.

Vitamin D-driving phosphate conserving mechanism in the small intestine and renal proximal tubules cells required activation of the sodium-phosphate co-transporters type IIb and type IIa correspondently. As a physiological counter player, parathyroid hormone (PTH) has the ability to internalise the Na/Pi-cotransporter Type IIa in the kidney, which decreases serum phosphate levels. Moreover renal activity of 1α-hydroxylase is also regulated by PTH in this way closing the feedback loop of phosphate endocrine regulation (Berndt et al. 2005).

Back in 1994, a case study was published, that described a patient with tumor-induced osteomalacia suffering on hypophosphatemia, low concentrations of 1,25(OH)2D3 and impaired reabsorption of phosphate in the proximal tubules cells, whereas the PTH level was normal. After removal of the tumor, her symptoms disappeared. Cultured tumor cells produced 10-30 kB proteins in the supernatant, which could selectively inhibit the Na/Pi-cotransporter in a PTH independent manner, as well as inhibit the activity of 1α-hydroxylase. These proteins where first described as “Phosphationins”, possible phosphate regulating hormons in the circulating bloodstream (Berndt et al. 2005; Q. Cai et al. 1994).

Today there is widely evidence that Fibroblast Growth Factor 23 (FGF23) acts as a main representative of the “Phosphationins” by down regulating both, the 1α-hydroxylase and the Na/Pi-cotransporter, which leads to a decrease in renal phosphate reabsorption. This molecule maintains its cell specific activity by using a co-receptor called Klotho (Urakawa et al. 2006). FGF23 is secreted by osteoblasts and osteocytes and may has its physiological role as a negative counter-regulatory hormone to vitamin D. Interestingly 1,25(OH)2D3 has a positive transcriptional effect on the bone FGF23 synthesis, which points to the feedback regulation of vitamin D level and therefore intestinal uptake of phosphate and calcium. Some studies reveal that serum phosphate is an additional regulator of the FGF23 bone expression, others could
not prove this connection (Quarles 2012). Nevertheless hypophosphatemia is believed to be the triggering mechanism behind chronic kidney disease associated increase in the soluble FGF23 level (Imanishi et al. 2004)

Additionally, Klotho is also regulated via vitamin D, through an (vitamin D responsive element (VDRE)) near the Klotho gene (Forster et al. 2011). In homozygote knockout mice (Kl⁻/⁻) the serum 1,25(OH)₂D₃ are highly elevated, which is believed to be due to the lack of FGF23 dependent down-regulation of 1α-hydroxylase (Yoshida et al. 2002). Therefore Klotho seems to be necessary in the kidney as a co-receptor for the canonical FGF23 pathway. However in extra-renal sites the importance of the Klotho co-receptor function for FGF23 remains unclear.

Recent studies suggest that in the endothelium FGF23 has a potential regulating function on the 1α-hydroxylase (Chanakul et al. 2013). However it was additionally shown that elevated FGF23 levels impaired vasorelaxation, due to an increase in superoxide levels ultimately leading to a decrease in bioavailability of NO (Silswal et al. 2014). This could be a triggering mechanism in cardiovascular diseases in chronic kidney disease (CKD) patients with chronically elevated FGF23 serum levels.

Klotho mutant mice (Kl⁻/⁻) and FGF-23 knockout mice (Fgf-23⁻/⁻) develop a similar phenotype proving that these two molecules are involved in the one signalling system (Kurosu et al. 2006). Klotho could be expressed in membrane-bound form which is defining as a co-receptor form for FGF23 ligand at FGF receptors (FGFR 1, 2, 3, 4) and a soluble factor generated by cleavage of the trans-membrane Klotho protein form. Several studies suggested soluble Klotho as a protective molecule in the cardiovascular pathology reducing endothelial oxygen stress molecules and preventing aortic calcification (M. C. Hu et al. 2011).

On the other hand increasing numbers of studies correlate soluble FGF23 level with negative outcomes in cardiovascular diseases and cardiac pathology (Faul et al. 2011; P. Hu et al. 2012; Jimbo and Shimosawa 2014). In haemodialysis patients with chronic kidney disease (CKD) elevated FGF23 trigged coronary artery calcification progression (Ozkok et al. 2013). However the role of FGF23 in vascular calcification is not conclusive (Zhu et al. 2013).
1.6. NO and ROS in the vascular system

In a physiological state the endothelium has the ability to sense shear stress due to alterations in the hemodynamic flow. This signal is transduced by mechano-receptors, a group of membrane proteins, located on the endothelial cell surface (Berk 2008; VanderLaan et al. 2004).

Several studies indicating that changes in a vascular blood flow contributes to an elevated level of glutathione (GSH) in the endothelial cells which leads to the shift in the reactive oxygen species (ROS; reactive oxygen species - a collective term for oxygen radicals such as \( \text{H}_2\text{O}_2 \), \( \text{O}_2^- \) or similar small molecules) production and regulation of the genes involved in the vascular tone maintenance (Berk 2008). There are links between vascular eNOS expression and increased endothelial ROS production in different pathologies (Förstermann and Münzel 2006; Y.-M. Yang et al. 2009).

Elevated ROS is associated with endothelial dysfunction (Matlung et al. 2009). Additionally elevated ROS levels can interact with NO to RNS (reactive nitrogen species), leading to the cell apoptosis and death (Hsieh et al. 2014). Increased cleavage of NO molecules to the RNS reduction NO bioavailability leading to increased vascular stiffness and impaired vascular tonus adaptations (H. Cai and Harrison 2000).

Also eNOS itself tends to produce less NO in the endothelium, when exposed with oscillatory shear stress. This mechanism, also mediated through limiting levels of L-arginin or the cofactor tetrahydrobiopterin (BH4), is known as eNOS uncoupling. Here the enzyme fail to produce NO, whereas superoxide is formed contributing to the oxidative stress level (Y.-M. Yang et al. 2009).

Several study link oxidative stress and arteriosclerosis (Yamano et al. 2015) and with endothelial dysfunction (Higashi et al. 2014). Interestingly, elevated FGF23 and decreased vitamin D serum levels were shown to associate with arteriosclerosis and endothelial dysfunction during CKD (Mirza et al. 2009). This observation points to the possible link between FGF23 and vitamin D signalling in endothelium modulating eNOS production. Moreover possible regulation of local vitamin D production by FGF23 through 1α-hydroxylase regulation could be not excluded.
Therefore, the aim of the present study was to explore the physiological and pathophysiological role of FGF23 and Klotho signalling in regulation of the vascular eNOS and 1α-OHase expression using genetically modified animal models.

1.7. Aim of this work

The current work is based on three, initially unrelated, lines of evidence. Firstly, we recently discovered that vitamin D is a regulator of eNOS, and that vitamin D receptor mutant mice (VDR\textsuperscript{Δ/Δ}) mice, characterized by a non-functioning VDR, have lower systemic NO levels and develop increased aortic impedance and arterial stiffness due to endothelial dysfunction (Andrukhova et al. 2014). Secondly, studies of others show that local production of 1,25(OH)\textsubscript{2}D\textsubscript{3} in endothelium may have potent biological effects. In particular, 1,25(OH)\textsubscript{2}D\textsubscript{3} produced locally in tissues by the enzyme 1α-OHase may act as an autocrine/paracrine modulatory mechanism. Endothelial cells express 1α-OHase and therefore endothelium is able to produce active vitamin D (Li et al. 2004). Thirdly, the work of others has shown that 1,25(OH)\textsubscript{2}D\textsubscript{3} can decrease endothelial superoxide production, and can protect human endothelial cells from oxidant injury through Erk1/2 signaling, also known as a key regulatory molecule in FGF23 action (Polidoro et al. 2013). However, it is currently unknown if FGF23 may also modulate the activity of endothelial 1α-OHase and, thus, local 1,25(OH)\textsubscript{2}D\textsubscript{3} production. In the current study we hypothesize that endothelial FGF23 signaling may modulate local 1,25(OH)\textsubscript{2}D\textsubscript{3} production in the endothelium which in turn modulates superoxide production, finally leading to the decrease in NO bioavailability. The aim of this study is to investigate the interaction between the FGF23 and 1,25(OH)\textsubscript{2}D\textsubscript{3} signalling in the regulation of endothelial function. The data obtained in this study will reveal whether extra-renal signalling mechanisms of FGF23 in endothelium contributes to pathology associated with the increase in circulating FGF23 levels like cardiovascular diseases, atherosclerosis, hypertension and chronic kidney disease. The results of this study may have important clinical implications, since could be easily translated into clinical medicine.
2. Material and Methods

2.1. Animals

Three-months old male and female mice kept under 24°C in a 12:12 hours light and dark cycle with free access to water and food. For RNA and protein expression analysis, four different animal genotypes were analysed: vitamin D receptor deficient (VDRΔ/Δ), VDR and Fgf23 double mutant (Fgf23+/-/VDRΔ/Δ) and VDR and Klotho double mutant mice (Klotho+/-/VDRΔ/Δ). Wildtype animals (WT) were used as a reference group. The mice were genotyped by PCR on genomic DNA isolated from tail tips. Fgf23+/-/VDR+/+ and Klotho+/-/VDR+/+ mutant mice on C57BL/6 background were interbred to generate WT, VDRΔ/Δ and compound Fgf23+/--/VDRΔ/Δ and Klotho+/--/VDRΔ/Δ mutant mice.

Due to a reduced intestinal uptake of calcium the VDRΔ/Δ animals develop secondary hyperparathyroidism when fed on normal diet. Therefore all animals were fed on a rescue diet starting from 16 days of age (Ssniff, Soest, Germany) containing 2.0% calcium, 1.25% phosphorus, 20% lactose, and 600 IU of vitamin D/kg. According to previous studies this normalises the calcium levels and prevents secondary hyperparathyroidism (Erben et al. 2002). It is known that single Fgf23+/-- and Klotho+/-- knockout mutants show severe phenotype including premature aging, dwarfism, reduced body weight, reduced insulin sensitivity (Streicher et al. 2012). The observed aging related phenotype in Fgf23- and Klotho-ablated mice is rather due hypervitaminosis D, resulting in hypercalcemia, and hyperphosphatemia, which was completely normalized in Fgf23+/+/VDRΔ/Δ and Klotho+/+/VDRΔ/Δ animals by feeding with the calcium and phosphorus rich rescue diet(Anour et al. 2012; Streicher et al. 2012). Therefore to reveal VDR-independent function of FGF23 we used Fgf23+/--/VDRΔ/Δ and Klotho+/--/VDRΔ/Δ compound mutants in the present study.

2.1.1. Mouse chronic renal failure model

For total nitrogen measurement aortas of mice undergoing we used a model for chronic kidney disease (CKD) induced by 5/6 nephrectomy (5/6 NX) in a 2-step process involving partial removal of the left kidney followed by right unilateral nephrectomy 7 days later (Massy et al. 2005). At the first step, the left kidney will be decapsulated to avoid ureter and adrenal damage, then the upper and lower poles will be partially resected via a left flank
incision. Bleeding will be controlled by electrical cauterization or microfibrillar collagen hemostasis (Avitene, Warwick, Great Britain). One week later in step 2, via a right flank incision the ureter, renal vein and artery of the right kidney will be ligated and the entire kidney will be removed and weighed. In the sham surgery control mice, a both flank incisions will be performed without renal tissue movement.

2.2. Dissection and tissue preparation

In this study the thoracal part of the aorta (*Aorta thoracalis*) was used for all types of the experiments. For mRNA and protein analysis animals were anaesthetised, aorta was dissected and snap-frozen in liquid nitrogen with subsequent long-term storage at -80°C. For aorta organ culture experiments, animals undergo euthanasia via cervical dislocation and aorta was collected. In brief, for only aortic dissection we opened the skin and chest cavity by cutting through the sternum, the diaphragm and alongside the rips. All organs in the thorax, obscuring the aorta, were removed, by cutting through the oesophagus and the trachea (Figure 1). The thoracal part of the aorta (visible alongside the spinal cord) was taken out by two clean cuts on the anterior and posterior side and immediately washed in cold Dulbecco’s Phosphate Buffered Saline (DPBS) (Gibco®, Invitrogen, Grand Island, NY). Remaining connective and fat tissue was removed from the aorta before further experiments.
2.3. RNA Expression

2.3.1. Homogenisation

For the mRNA isolation the samples were homogenised in liquid nitrogen, using a Mixer Mill (Retsch MM200 Mixer Mill, Haan, Germany) with a frequency of 30x 1/s for 20 seconds. The homogenates were subsequently resuspended in 500 µl TRIzol® Reagent (Ambion®, Austin, USA) as described previously by Sacchi and Chomczynski (Chomczynski and Sacchi 1987).

2.3.2. RNA Isolation

In brief, for the mRNA isolation a chloroform – isopropanol - ethanol precipitation strategy was used as described (Chomczynski and Sacchi 1987). Instead of chloroform the less toxic alternative 1-bromo-3-chloropropan (BCP) was used. All steps followed after the

Figure 1: Dissection of the aorta thoracalis.

After euthanasia, the mouse was placed at the backside (a) and the skin was opened with a sagittal cut from the cervical ribs to the linea alba. Then the thorax was opened by cutting alongside the ribs, through the diaphragm and the sternum (b). In (c) all obscuring organs were removed, including lungs, trachea, oesophagus and heart.
manufactures protocol (Ambion 2012). Finally, mRNA pellet was suspended in 10µl nuclease-free water (Ambion® DEPC-Treated Water, Austin, USA).

2.3.3. RNA Integrity and Quantification
RNA concentration was determined with UV-Vis spectrometry, using the Nano Drop® 2000 device (absorption at 260nm - 1 µl RNA droplet) (Thermo Scientific, Wilmington, USA). One microgram (in other experiments two microgram) of mRNA was inserted for first-strand cDNA-synthesis. RNA integrity was measured with Agilent® technology (Bioanalyser 2100, Agilent® Technologies, Santa Clara, USA) according to the manufactures protocol (Agilent Technologies 2007).

2.3.4. Reverse Transcription
For first-strand cDNA-synthesis we used the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA), according to the manufactures protocol (Biosystems 2010). No RNAse inhibitor was used in the first series of experiments. For the following experiments RNA-ase inhibitor was used and DNA-digestion was performed before the reverse transcription procedure (RiboLock RNase-inhibitor; DNase I, RNase-free (supplied with MnCl2), 1 U/µL), Thermo Scientific, Wilmington, USA) at 37 °C for 30 min, followed by a 10 min 65°C incubation with 1µl 50mM EDTA, to inactivate the enzyme.
### 2.3.5. Primer Design

In this study we following primers were used:

<table>
<thead>
<tr>
<th>Gene/symbol</th>
<th>Exon</th>
<th>Amplicon Length</th>
<th>Annealing Temp</th>
<th>Forward Primer Length</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Length</th>
<th>Reverse Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp27b1</td>
<td>Exon 1</td>
<td>68bp</td>
<td>60 °C</td>
<td>17bp</td>
<td>5’ TCCCTGCAGCGGATCCT</td>
<td>17bp</td>
<td>5’ GTGCCGCTTGCGTTTTC C3’</td>
</tr>
<tr>
<td></td>
<td>Exon 11/12</td>
<td>77bp</td>
<td>60 °C</td>
<td>22bp</td>
<td>5’ GCATCACCCAGGAAGAAC</td>
<td>19bp</td>
<td>5’ CATCACCGTGCCCCAT GAGT 3’</td>
</tr>
<tr>
<td></td>
<td>Exon 7/8</td>
<td>79bp</td>
<td>60 °C</td>
<td>29bp</td>
<td>5’ GACAGAGACATCGGTTG</td>
<td>22bp</td>
<td>5’ CCCTTGAAGTGCCAT AGTGACA 3’</td>
</tr>
<tr>
<td></td>
<td>Exon 2/3</td>
<td>60bp</td>
<td>60 °C</td>
<td>16bp</td>
<td>5’ CGCCACACCGTGCTT</td>
<td>19bp</td>
<td>5’ TGGAGCCCTGCGCCA TTCT 3’</td>
</tr>
<tr>
<td></td>
<td>Exon 5/6*</td>
<td>103bp</td>
<td>60 °C</td>
<td>20bp</td>
<td>5’ TGGAGTGGACACGGGAT</td>
<td>21bp</td>
<td>5’ GGTCACACCTGTGAT CTCAGA 3’</td>
</tr>
</tbody>
</table>

**Table 1: Primer Sequences**

Primer length and sequences, annealing temperatures and amplicon length

* Primerpair was designed with corresponding TaqMan®-Probe (Table 2)

Primers were predesigned from other members of our group, with the exception of the last primerpair (Cyp27b1 Exon 5/6) which was newly designed with a corresponding TaqMan®-probe.

For the design of the primers and the probe we used the **Primer Express 2.0®** (Applied Biosystems, Foster City, USA) and **Perlprimer 1.1.20** (http://perlprimer.sourceforge.net) for Windows® XP (Marshall 2004; Singh and Pandey 2015). Candidate Sequences were further checked for their specificity with the **Primer-BLAST Software** (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye et al. 2012). The Tool NETPrimer (PREMIER Biosoft, Paolo Alto, USA) was than used to calculate the Gibbs free enthalpy (ΔG) for primer dimers and cross dimer formation, as well as hairpin loops (“NetPrimer
Manual” 2009). Lastly we wanted to look for secondary structures of the amplicon and therefore used the “The mfold- Web Server” (http://mfold.rna.albany.edu/?q=mfold) (Zuker 2003). For estimation the mRNA expression Oaz1 gene were used as a housekeeping gene (Meyer et al. 2013).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Oligonucleotide</th>
<th>Temperature</th>
<th>Length</th>
<th>Sequence</th>
<th>Cross-dimerization</th>
<th>Hairpin structure</th>
<th>Primer dimerization*</th>
<th>Amplicon secondary structure*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp27b1 (Exon5/6)</td>
<td>Forward primer</td>
<td>60 °C</td>
<td>20 bp</td>
<td>5’ TGGAGTGGACACGATTCAC 3’</td>
<td>ΔG = -4.64 kcal/mol</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>60 °C</td>
<td>21 bp</td>
<td>5’ GGGCCAGCTGATGCTACGA 3’</td>
<td>ΔG = -0.64 kcal/mol</td>
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</tr>
<tr>
<td>TaqMan®-Probe</td>
<td>70 °C</td>
<td>27 bp</td>
<td></td>
<td>5’ TCGGGAAGGCTCCGTTCCAGTC 3’</td>
<td></td>
<td></td>
<td></td>
<td>ΔG = -2.57 kcal/mol</td>
</tr>
</tbody>
</table>

Table 2: Primerpair Cyp27b1 (Exon5/6) information

Primer and TaqMan®-probe information for primerpair Cyp27b1 (Exon5/6). The probe was marked with a FAM (Carboxyfluorescein) fluorophore.
* Not all data shown, only the lowest value in this category.

2.3.6. Quantitative PCR

The cDNA samples were 1:10 diluted for quantification with the real-time PCR Corbett Rotor-Gene 6000 (Quiagen Corbett Life Science, Australia) using HOT Firepol® EvaGreen® Mix polymerase (Solis Biodyne, Tartu, Estonia) with Primers in 0,3 µM concentration. EvaGreen® is a DNA-intercalating dye, that shows low tendency for unspecific binding due to its low affinity for ssDNA and short DNA-fragments (Mao et al. 2007). For TagMan®-probe based assay 0,15 µM of the FAM-probe and 1 µM of MgCl2 were added to the mastermix, using a HOT Firepol® mix without EvaGreen® (Solis Biodyne, Tartu, Estonia). One microliter of the ten-fold diluted cDNA was used as a template per reaction. The experiment was performed as a doublet reaction, which means every sample was used for two reactions.

Necessary negative controls (non-template control, RT − Controls) and positive controls (endogenous and exogenous control) were used to validate the assay. We used a standard curve to calculate the efficiency of the new primer pairs. With genomic mouse DNA the assay was validated for unspecific DNA quantification.
For quantification we programmed the machine with following parameters:

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
<th>Repeats</th>
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</thead>
<tbody>
<tr>
<td>Hold</td>
<td>95° C</td>
<td>15 min</td>
<td>x1</td>
</tr>
<tr>
<td>Cycle</td>
<td>95° C</td>
<td>15 sec (with TaqMan®) 30 sec</td>
<td>x45</td>
</tr>
<tr>
<td></td>
<td>60° C</td>
<td>50 sec</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3: Parameters for qPCR Cycling**

Samples were quantified in a real-time PCR Corbett Rotor-Gene 6000 Machine. TaqMan® samples were cycled at 95 °C for 15 sec, whereas other primers were cycled for 30 sec in this step.

### 2.3.7. Analysis ($2^{ΔΔCt}$ – Method)

We measured and calculated relative RNA-expression by using the modified Pfaffl method ($2^{ΔA}$-method). Therefore we selected a reference gene (Oaz1), a so-called housekeeping gene, as it is invariably and constantly expressed in this tissue and was described to be stably expressed (Radonić et al. 2004). The relative gene expression was measured by comparing the difference in the Ct-Values of reference and target gene to the wild type group (or vehicle group) (Pfaffl and Pfaffl 2001).

1. \[ ΔC_{[control]} = C_{H;control} - C_{A;control} \]
2. \[ ΔC_{[sample]} = C_{H;sample} - C_{A;sample} \]
3. \[ 2^{ΔΔCt} = 2^{ΔC_{[sample]} - ΔC_{[control]}} \]

**Equations 1-3: Calculation of the $2^{ΔΔCt}$ – values**

For calculation of the $2^{ΔΔCt}$ – method four $C_t$ – values are obtained: (1) $C_t$ – value of the housekeeping gene (H) in the control group ($C_{H;control}$), (2) $C_t$ – value of the target gene (A) in the control group ($C_{A;control}$), (3) $C_t$ – value of the housekeeping gene (H) in the sample group ($C_{H;sample}$), (4) $C_t$ – value of the target gene (A) in the sample group ($C_{A;sample}$) to calculate the $ΔC_t$ values. $2^{ΔΔCt}$ values can be calculated via log-transformation as seen in equation 3.
2.4. Aortic Rings Organ Culture

For aortic rings organ culture aorta was dissected from the 3-month-old male WT mice and immediately (after washing with cold DPBS) treated for 24h at 37°C and 5% CO₂/95%. The aorta from one animal was divided in six equal pieces and three parts were used for one well, respectively. For organ culture cultivation a commercial Dulbecco’s Modified Eagle Medium (DMEM) (Gibco®, Invitrogen, Grand Island, NY) was supplemented, by adding additional 10% fetal bovine serum (FBS), 2% penicillin and streptomycin (DMEM+ 10%FBS+2%PS).

Following treatment were applied for the aortic organ culture: (1) vehicle (DMEM+10%FBS+2%PS only), (2) 100ng/ml recombinant FGF23, (3) 100µM Apocynin (Acetovanilllon) dissolved in DMSO (Dimethylsulfoxide), (4) 10 mM N-Acetyl-Cystein, (5) 100ng/ml recombinant FGF23 and 100µM Apocynin, (6) 100ng/ml recombinant FGF23 and 10mM N-Acetyl-Cystein.

Following 24h of incubation the samples were homogenised mechanically by using Scienceware® Micro Centrifuge Sample Pestles (Thomas Scientific, Swedesboro, USA) and the mRNA were isolated using TRIzol® Reagent.

2.5. Protein Expression

2.5.1. Protein Isolation and Quantification

For eNOS protein expression we isolated total protein from the aorta thoracalis of three month-old male animals. Aorta was homogenised using liquid nitrogen in the Mixer Mill (Retsch MM200 Mixer Mill, Haan, Germany) at 30x 1/s frequency for 40 seconds with 200 µl radioimmunoprecipitation assay buffer (RIPA buffer). Buffer contained 50 mM Tris HCl pH 8, 150 mM NaCl, 1% Triton X100, 0,5% sodium deoxycholate and 0,1% SDS. Protease and phosphatase Inhibitors (PI) were added to the buffer, prior the usage.

The homogenates were treated with ultrasound with the Sonifier B12 (Branson, Danbury, USA) for approximately seven seconds to disrupt the DNA in the solution. The samples were centrifuged at 2000g for 10 min at 4°C and protein quantification was performed using supernatant by bicinchoninic acid (BCA) based assay (Pierce™ BCA Protein Assay Kit,
Thermo Scientific, Wilmington, USA). Albumin standard curve (bovine serum albumin (BSA)) was used for concentration calculation of the unknown samples.

For western blotting analysis 50µg/well total protein of the aorta homogenate were used. Prior loading on the gel, the samples were incubated at 95 °C for 10 min to denature the proteins. Gradient polyacrylamid-gels 4-20% (Bio-Rad, Hercules, USA) were used for electrophoresis with Prestained Protein PageRuler™ Ladder marker (10 – 250 kDa) (Thermo Scientific, Wilmington, USA). SDS-Page was run 30min at 80V and then 2h at 120V using running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS).

Gels were semi-dry blotted on nitrocellulose membranes (Thermo Scientific, Wilmington, USA) with a standard transfer buffer (48mM Tris, 39mM glycine, 0.04% SDS, 20% methanol). Subsequently, the membrane was blocked, using a blocking buffer (2% BSA in TBS-T Buffer (150 mM NaCl, 10 mM Tris (pH 7.4/HCl), 0.2% (v/v) Tween-20)). The primary antibodies: (1) polyclonal antibody anti-eNOS, rabbit, (1:1000 in blocking buffer, Thermo Scientific) and (2) polyclonal antibody anti-β-actin, mouse (1:5000 in blocking buffer, Sigma) were incubated over night at room temperature, respectively. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 in blocking buffer, Amersham Life Sciences). ELC kit (Amersham Life Sciences) were used for visualisation of the specific protein bands. β-actin was used as a loading control.

To quantify eNOS relative protein expression Image Quant 5.0 software (Molecular Dynamics) was used.

2.6. Nitric Oxide metabolites measurement

2.6.1. Sample preparation and Homogenisation

For total nitric oxide measurement we prepared an iPBS (isotonic PBS) homogenisation solution (PBS + 10mM NEM (N-Ethylmaleimid) + 2,5mM EDTA), that inhibit a degradation of endogenous RSNOs and nitrate as well as blocking proteins SH-Groups and
preventing artificial nitrosation (Bryan and Grisham 2007; Bryan et al. 2005; Marley et al. 2001).

Due to the small amount of tissue, all aortas of one group (three organs for the CKD WT group and two organs for all other groups) were pooled together to one sample, leading to (n=4) individual samples, used for further investigation. We weight the tissue prior homogenisation and performed 10-fold dilution (or 15-fold for CKD WT and CKD Sham aortas) with the homogenisation solution before usage. This strategy was established in the prior pilot experiments where the dilution factors and pooling strategy were previously optimised. Total nitric oxide content were normalised to the wet aorta weight.

Samples were mechanically homogenised with a rotating teflon tissue pestle (2.5 ml glass jar) (Thomas Pestle Tissue Grinder, Thomas Scientific, Swedesboro, USA) until most of the tissue pieces were dissolved. After centrifugation the supernatant was collected for total nitric oxide measurement and haemoglobin quantification.

2.6.2. NO-Analysis

For total nitric oxide measurement an ozone – chemiluminescence based technology (NO Analyser 280i Sievers, Boulder, USA) was used. An O₂ tank served as a source of O₃, whereas an inert gas (N₂) was used as a carrier of NO to the detection chamber. Samples can be directly injected into a bubbling reducing agent (0.2g Vanadium(III)chlorid in 25ml 1M HCl), where nitrate, nitrite and nitrothiol species are reduced to NO, as described in Bryan & Grisham. Arising NO can be quantitatively measured upto nano or pico molar concentrations according to the manufacturer and are representative for the total nitric oxide concentrations in the homogenate. Differences in the total nitric oxide amount among the animal groups, could be an indicator of elevated or reduced NO production.

The whole reaction was conducted in hot temperature. For that 95°C water bath was used to preheat the reaction chamber and the vanadium(III)chlorid. By reduction, nitrate and other nitrogen species are converted into NO (Braman and Hendrix 1989), which was captured in the detection chamber of the device. Every sample was measured twice to increase the reproducibility.
2.6.3. Haemoglobin measurement

As a haeme-containing proteins are the major sources for nitrate destruction *in vivo* (Piknova and Schechter 2011) and the aortas may contain some blood during homogenisation, we measured correlation between the haemoglobin concentration and total nitric oxide concentration in our samples. Therefore, we used the same samples (n=4) as for NO measurement to quantify the haemoglobin concentration. Thus spectroscopic analysis (at 415nm) was performed, using a reference absorption value to calculate the sample haemoglobin concentration.

2.6.4. Quantification of total nitric oxide

The area under the peak of the injection in the NO-Analyser was used to quantify the total nitric oxide concentration in the sample. Areas under the curve were corrected with values from a standard, the buffer and water. A NaNO₂ standard curve was used to calculate the concentration in µM.

2.7. Statistics

For calculations and graphical presentation of the data, Microsoft® Excel® 2011 (Version 14.5.2) for Mac and R version 3.0.2 (2013-09-25) (http://www.R-project.org/) were used. The data was analyzed by one-way analysis of variance (ANOVA) and is presented mean and SD of 4-6 animals per group or 3 replicates in the organ culture experiments respectively. The resulting 2ΔΔ-values were tested for significance in difference between the genotypes with a two-sided, two-sample t-Test, with a 95% confidence interval.
3. Results

3.1. eNOS mRNA expression is regulated in *Fgf23<sup>-/-</sup> VDR<sup>Δ/Δ</sup> and *Klotho<sup>-/-</sup> VD<sup>Δ/Δ</sup> compound mutant mice

Study of Silswal et. al, describe that aortic rings treated with high concentrations of FGF23 show impaired endothelial mediated contractility and lead to NO bioavailability decrease in the endothelium. Additionally authors show that low aortic NO levels are not due to the changes in eNOS phosphorylation (on Thr<sup>495</sup>), but rather origins from elevated superoxide levels (Silswal et al. 2014). On the other hand, active form of vitamin D, 1α,25-dihydroxyvitamin D3 positively modulates endothelial NO production, and that VDR-ablated mice show impaired endothelial functions and increased aortic stiffness. It is also well-known that FGF23 is one of the main regulators of the 1,25(OH)<sub>2</sub>D<sub>3</sub> production in the kidney, acting through inhibition of the 1α-hydroxylase. Study of Silswal et. al, show that all four FGF-receptor types (Fgfr1, Fgfr2, Fgfr3 and Fgfr4) possibly mediating FGF23 signalling are expressed in the mouse aorta. Expression of a co-receptor for FGF23 axis α-Klotho in membrane and soluble form at the vasculature remains still contradictive (M. C. Hu et al. 2011; Lindberg et al. 2013). The effect of FGF23 on vascular NO production was shown to be α-Klotho independent (P. Hu et al. 2012). Therefore, the regulation of the interaction between the FGF23/Klotho and 1,25(OH)<sub>2</sub>D<sub>3</sub> signaling axis in the local production of 1,25(OH)<sub>2</sub>D<sub>3</sub> through endothelial 1α-OHase is still pour understood.

In order to reveal if FGF23/Klotho signalling could influence eNOS endothelial expression we first tested if eNOS mRNA expression was changed in the aortas of WT, VDR<sup>Δ/Δ</sup> and compound *Fgf23<sup>-/-</sup>/VDR<sup>Δ/Δ</sup> and *Klotho<sup>-/-</sup>/VDR<sup>Δ/Δ</sup> mutant mice on the rescue diet used in the present study.
The qPCR data demonstrate significantly increased eNOS mRNA expression in aortas of Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) compound mutant mice (p < 0.05) and significantly reduced eNOS mRNA expression in aortas of Klotho\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) compound mutant mice (p < 0.05) as compared to the VDR\(^{\Delta/\Delta}\) and WT animals (Figure 2). Interestingly, an increase in eNOS mRNA expression in Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) compound mutants was significantly higher as compared to Klotho\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) compound mutant mice (p < 0.05). Moreover, in accordance with previous study, (Andrukhova et al. 2014) we found slight but not significant decrease for eNOS mRNA expression in VDR\(^{\Delta/\Delta}\) mutants as compare to the WT animals at 3-month-old age (Figure 2).

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**Figure 2:** The relative expression of eNOS in the aorta thoracalis of different mutant mice models

Here we examine the relative mRNA Expression of Nos3 in the aorta thoracalis. WT (n=15), VDR\(^{\Delta/\Delta}\) (VDR, n=12), Klotho\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) (KV, n=13) and Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) (FV, n=12) represent the different mutant mice we used in this study. Negative reverse transcription controls, non-template controls and showed no or very low amplification ($C_T$-values >35), which is an indicator for the specificity of the assay (data not shown). We used a two-sided, two-sample t-Test to determine significant differences of VDR, KV and FV compared with wt (*** p< 0.01). Median and 25%- and 75%-quantile are shown in the boxplot (left). Means ± SEM are shown in the barplot (right).

**Figure 3:** Western Blot analysis of aorta eNOS protein expression in WT, VDR\(^{\Delta/\Delta}\), Klotho\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) and Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) mice.

Original Western Blot images of eNOS and protein expression in aorta of WT (n=3), VDR\(^{\Delta/\Delta}\) (VDR, n=3), Klotho\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) (KV, n=3) and Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) (FV, n=3) animals. β-actin protein expression was used as a loading control for quantification of the Western Blot.

03.03.2015
3.2. Aorta eNOS protein expression is regulated by FGF23/Klotho signalling

Posttranscriptional modifications are involved in regulating mRNA stability, translational efficiency or localisation and transport. It was described, that eNOS posttranscriptional modifications play an important role for the protein functionality (Searles 2006). eNOS primary transcript could be changed in the cis- and trans- elements within the 3’ untranslated region (3’UTR) (Searles 2006). There are reports indicating that eNOS protein also could be modified by antisense mRNA-strand (called sONE), derivate from a transcriptional unit on the opposite DNA strand of the Nos3 gene and finally reduce the eNOS activity in non-endothelial cell (Robb et al. 2004).

Therefore, aortic eNOS mRNA expression change in Klotho−/−/VDRΔ/Δ and Fgf23−/−/VDRΔ/Δ compound mutants mice were validated by Western blotting analysis in order to analyse eNOS protein expression (Figure 3). Samples were normalised to β-actin, a loading control, to carry out relative protein amounts. Here we could observe a slight regulation among the genotypes, as band intensity shows inhomogeneity.

Western blotting analysis demonstrate that Klotho−/−/VDRΔ/Δ and VDRΔ/Δ animals show reduced eNOS protein amounts in the aorta thoracalis as compared to WT mice (Figure 4). These findings are in a good agreement with the eNOS mRNA expression data observed in these animals. Interestingly, in aortas of Fgf23−/−/VDRΔ/Δ compound mutants decreased eNOS protein expression was observed as compared to the other animal groups (Figure 4).
3.3. Measurement of total Nitric Oxide content in the Aortas of the Klotho\textsuperscript{-/-}/VDR\textsuperscript{Δ/Δ} compound mutants mice and in the mouse chronic kidney disease model.

It is known from the previous studies that eNOS enzyme is known to react with cofactors such as tetrahydrobiopterin (BH4), which could lead to superoxide production instead of nitric oxide (Y.-M. Yang et al. 2009). In this case, an increase in aortic eNOS protein expression would not lead to the increase in NO production, but could rather contribute to the extensive endothelial superoxide production. According to Silswal et. al, 2014, treatment of the aortic rings with high concentrations of recombinant FGF23 decreases NO bioavailability by increasing superoxide levels \textit{in vitro}. On the other hand it is known, that in CKD patients with chronically elevated circulating FGF23 levels, observed higher prevalence of endothelial dysfunction which is possibly linked to the elevated endothelial oxidative stress described in these patients (Mirza et al. 2009). If FGF23 could directly induce changes in the aortic NO production and if Klotho could play an important role for these regulation remains still unidentified.

Figure 4: Quantification of the eNOS relative protein expression in aorta of WT, VDR\textsuperscript{Δ/Δ}, Klotho\textsuperscript{-/-}/VDR\textsuperscript{Δ/Δ} and Fgf23\textsuperscript{-/-}/VDR\textsuperscript{Δ/Δ} compound mutants mice.

Quantification of the Western blotting analysis of the aortic eNOS protein expression in WT, VDR\textsuperscript{Δ/Δ}, Klotho\textsuperscript{-/-}/VDR\textsuperscript{Δ/Δ} and Fgf23\textsuperscript{-/-}/VDR\textsuperscript{Δ/Δ} compound mutants mice. Image J software. The β-actin expression was used as a loading control. Data presented as means ± SEM are shown in the barplot (* p< 0.05 vs. WT ).
Since it was described previously that in VDR $\Delta/\Delta$ mice the level of total nitrate in the serum is impaired (Andrukhova et al. 2014), in the next step of our study we measured the total nitric oxide amount in the aorta homogenates of 2-month-old male WT and $Klotho^{+/}/VDR^{\Delta/\Delta}$ animals. Using chemiluminescence based technology described by Bryan & Grisham we determine the concentration of nitric oxide, nitrate, nitroso and nitrothiol species, in the homogenates prepared from the frozen aortas (Bryan and Grisham 2007). The data where obtained as quotient of detected total nitric oxide (in $\mu$M) normalised to the wet weight of the aorta (in g), (Figure 5). No difference was detected in total nitric oxide content in aortas of $Klotho^{+/}/VDR^{\Delta/\Delta}$ animals as compared to WT animals (Figure 5). It is published previously that $Klotho^{+/}/VDR^{\Delta/\Delta}$ mutants has elevated serum FGF23 level (Anour et al. 2012). In addition other study shows that in VDR $\Delta/\Delta$ animals is lover endothelial NO production takes place through downregulation of eNOS expression (Andrukhova et al. 2014). Moreover it was shown that recombinant FGF23 could regulate endothelial NO production by increasing superoxide production (Silswal et al. 2014). Therefore, we hypothesise that unchanged aortic total NO level observed in $Klotho^{+/}/VDR^{\Delta/\Delta}$ compound mutants could be driven by elevated FGF23 level in this animals thus causing the normalising in aortic NO level observed VDR $\Delta/\Delta$ mice to those of WT. This data points to the possible Klotho-independent involvement of FGF23 signalling in the regulation of aortic NO production.

To further explore if FGF23 signalling could be involved in NO production we measured total nitrogen amount in the aorta homogenates of 5-month-old male CKD or Sham operated WT mice at 8-weeks post-operation. It is described, that mice with 5/6 nephrectomy-induced CKD at 8-weeks develop progressive CKD and has increased circulating FGF23 level correlating with the decreased renal functions. Therefore, mouse CKD model is an additional representative to examine the effect of excessive FGF23 level on aortic NO production during CKD pathology. Interestingly, we observed 9.5-fold higher total NO production in both CKD and Sham-operated groups as compared to the WT (Figure 5). Moreover, we found decreased total aortic NO level in CKD-operated animals as compared to the Sham-operated mice. The difference in total NO content between the two experiments could be explained by different experimental settings including animal age and possible effect of the animal medical treatment following CKD, or inflammatory responses.
Since it is known that NO is available in blood and that NO reacts with oxyhemoglobin to produce methemoglobin, with ferric iron and nitrate ions. Recent work suggests that most of the methemoglobin circulating in red blood cells is derived from this oxidation process. As haeme-containing proteins are a major source for nitrate destruction \textit{in vivo} (Piknova and Schechter 2011) and the aortas contained a reservoir of blood when used for homogenisation, there could be a relation between haemoglobin concentration and total nitrogen concentration.

Therefore, to exclude the possibility that the residues of the blood in the aorta samples used for the measurements could influence the total nitrate quantities results of our study, we

**Figure 5:** Total nitric oxide content in aortas of WT and \textit{Klotho}⁻⁻⁻/VDRΔΔ animals as well as in CKD animals.

A) Total nitric oxide content in aortas of WT and \textit{Klotho}⁻⁻⁻/VDRΔΔ (KV) animals. No changes were found between the animal groups.

B) Total nitric oxide content in aortas of CKD and Sham animals. Lower contents were found in CKD animal group. The numbers (n) represents the animals used for analysis. Mean value of two replicates is plotted on the graphic.
performed measurement of the total nitric oxide content in supernatant of the aorta homogenate used for the total aortic tissue NO content quantification.

Interestingly, we were not able to find a positive correlation between haemoglobin and the total NO in the settings of the present measurements ($R^2 = 0.7717$, $n=4$, $p > 0.05$) (Figure 6). This data suggest that the blood content of the aorta organ samples did not distort the results obtained in the previous chapter.

![Figure 6: Correlation between haemoglobin and total nitric oxide concentration.](image)

The total nitric oxide content of the total aorta homogenate supernatant samples ($n=4$) did not significantly correlated with the haemoglobin concentration. The red line indicates the hypothetical regression between haemoglobin values and total nitrogen content normalised to the wet aorta weight. Bars at the axis, represent the potential median and quantile values.
3.4. FGF23 regulation of Cyp27b1 (1α-OHase) expression in aortic rings organ culture is dependent on the intracellular ROS and NO levels.

One key hypothesis of this work is that FGF23 could decrease bioavailability of NO by increasing endothelial superoxide (Silswal et al.) and that the local production of vitamin D₃ via endothelial 1α-hydroxylase (1α-OHase) could be involved in this process. Therefore, to prove this hypothesis we treated in vitro organ culture of aortic rings dissected from WT mice with recombinant FGF23 (100 ng/ml) alone or in combination with the ROS inhibitor Apocynin, an inhibitor of NADPH-Oxidase (Wang et al. 2015), and a scavenger for NO N-Acetyl-Cystein (Tepel et al. 2003) for 24h and then collected the samples for 1α-OHase mRNA expression analysis.

The data show that recombinant FGF23 as well as Apocyanin or N-Acetyl-Cystein treatment alone didn’t show any regulatory effect of 1α-OHase mRNA expression (Figure 7). Interestingly, combined treatments with recombinant FGF23 and Apocyanin and recombinant FGF23 and N-Acetyl-Cystein decreased 1α-OHase mRNA expression in aorta organ cultures 24 h post-treatment (Figure 7). Despite the results did not reach the statistical significance because of the low animal number per group used (n=2), the result of the present experiment clearly suggest that endothelial ROS and NO production modulates FGF23 signalling regulation of aortic 1α-OHase expression.
3.5. Validation of the Cyp27b1 (1α-OHase) mRNA expression analysis in aortas of WT, VDRΔ/Δ, Klotho-/-/VDRΔ/Δ, and Fgf23-/-/VDRΔ/Δ animals.

It is well described that FGF23 is able to regulate renal 1α-OHase expression a key molecule in the vitamin D production at the transcriptional level (Chanakul et al. 2013). Vascular 1α-OHase expression and local production of vitamin D3 in the endothelium is well described (Daniel Zehnder et al. 2002b). The group of Zehnder et al. identified that regulation of this gene may occur through inflammatory molecules. In other studies FGF23 was mentioned as possible candidate for regulation of 1α-OHase expression in the vascular endothelium (Chanakul et al. 2013). Based on this, in the next experiment of our study we examine the relative expression of 1α-OHase in the aorta thoracalis of WT, VDRΔ/Δ, Klotho-/-/VDRΔ/Δ, and Fgf23-/-/VDRΔ/Δ compound mutant animals. Since the Ct-values obtained in qRT-PCR analysis show very low values (< 30 cycles) and were not statistically significant we designed the new primers with a TaqMan®-probe to optimise the assay. Technical improvements such as DNase digestion and RNase inhibiting during reverse transcription were additionally used to increase the specificity. Furthermore the RNA integrity was measured using the Agilent
technology. Out of 45 samples 17 showed a RIN value > 5.5: an indicator of the RNA quality. Used RT-control samples for the assay validation, we excluded that neither the DNase digestion, nor the reverse transcription procedure could affect $\alpha$-OHase expression in the used samples. Interestingly, after exclusion of the samples with a low RIN value, the C$_T$-values results for $\alpha$-OHase expression still remains > 30 cycles. This data suggested that the factor other than the RNA quality of the samples may influence $\alpha$-OHase expression and stability in the sample. Interestingly compared to the aorta $\alpha$-OHase mRNA expression data from *in vivo* experiment using WT, VDR$^{\Delta/\Delta}$, Klotho$^{-/-}$/VDR$^{\Delta/\Delta}$ and Fgf23$^{-/-}$/VDR$^{\Delta/\Delta}$ compound mutant animals we observed very consistent expression of the $\alpha$-OHase mRNA expression in the organ culture experiments shown in the previous chapter. Also the RIN values of the samples were around 10 and the C$_T$-values were in the applicable range. This leads us to the conclusion that that aortic $\alpha$-OHase mRNA expression could be regulated by additional mechanism that leads to either artificial upregulation of $\alpha$-OHase mRNA in the organ culture due to the lack of substrate, 25(OH)$_2$D$_3$ *in vitro*, or to by specific degradation stimulated during aortic sample dissection due to the longer post-mortem interval (PMI) and possible endothelial sensitivity to the hypoxic condition. Further experiments should be performed in order to validate the exact mechanisms regulating endothelial $\alpha$-OHase mRNA expression stability.
4. Discussion

4.1. FGF23 regulates eNOS expression in aorta

The role of FGF23 on the endothelium has been widely discussed. Here we provide evidence that a systemic lack of FGF23 increases the expression of eNOS in the VDR-independent fashion. Interestingly it was previously shown that lack of VDR signalling in VDRΔ/Δ animals lead to the lower eNOS expression and decreased endothelial NO production (Andrukhova et al., 2014). These results implicate that FGF23 and vitamin D signalling may act as a counter player in eNOS expression regulation. These findings are in agreement with the study of Silswal et al., who demonstrated a decrease of NO bioavailability after the high dosage of recombinant FGF23 treatment leading to the impaired aortic agonist-induced contractility.

The possible converge-point for the FGF23 and vitamin D signalling in regulation of endothelial eNOS expression could be the Erk 1/2 kinase signalling pathway since both molecules were shown to activate the intracellular mediator. It is demonstrated, that Erk1/2 phosphorylation stimulate DNA binding proteins (HDAC1), which are located in the promoter region of the Nos3 gene and triggered eNOS transcription (D. Yang et al. 2012). It is also well described that classical FGF23 signalling involves Erk1/2 phosphorylation in proximal tubule cells to regulate phosphate reabsorption process as well as in the smooth muscle cells where it was suggested to be involved in the regulation of the vascular calcification during CKD (Zhu et al. 2013). Based on these evidences, decreased eNOS expression would be expected in the FGF23 ablated mice. Surprisingly, data of the present study show that lack of FGF23 signalling rather induce eNOS mRNA and protein expression levels in absence of VDR signalling, suggesting reciprocal FGF23/vitamin D regulation of eNOS expression in aorta. Beyond the mRNA regulation there is a possible phosphorylative inactivation of the eNOS protein triggered by Erk1/2 (see Figure 8) (Salerno et al. 2014)

Vitamin D is also able to stimulate Erk 1/2 in a tissue specific manner, mainly determined by the tissue specific RXR subtype (Narayanan et al. 2004). Active VDR-RXR complexes can activate Erk1/2 by phosphorylation and alters eNOS transcription (Molinari et al. 2011). It was indicated previously that endothelial cells as well as smooth muscle cells express VDR. Since eNOS mRNA and protein expression in the present study were examined using the
whole aortic organ including endothelial cell, smooth muscle cells, and possibly blood and connective tissue, one could not exclude the possibility of the cell specific VDR effect. Therefore, the exact mechanism of FGF23 and vitamin D interaction in the endothelial cells still remains elusive and need further investigation.

It was postulated that elevated serum FGF23 levels lead to the increase in superoxide production in the endothelial cell due to uncoupling of the eNOS enzyme (Silswal et al.). Circulating FGF23 known to be elevated in CKD patients and correlate with the disease progression and pathology (Jimbo and Shimosawa 2014). It is also shown that superoxide production associated with and CKD progression and endothelial dysfunction in these patients. Here we provide evidence that total nitric oxide levels are slightly reduced in CKD mouse model, as compared to the sham animals. This data suggest that elevated circulating FGF23 may lead to the reduced NO bioavailability due to the possible superoxide production increase. Interestingly, sham animals showed a ten fold higher concentration in nitric oxide species, compared to WT. As operation lead to possible postsurgical inflammatory response, this could influence the nitric oxide levels in a positive way. It was reported previously that inflammation could lead to higher expressions of Cyp27b1 and Vdr (Adams and Hewison 2012), which is believed to change local NO production (Andrukhova et al. 2014). However, further experiments have to be performed to validate this hypothesis.

Moreover, elevated superoxide production is suggested to occur after uncoupling of the eNOS. Dysfunction of the urea cycle can lead to limiting levels of L-arginine in endothelial cells, an amino acid, which is used as an NO donor. As low availability of L-arginine, especially caused by arginases (I and II) acting as a substrate competitor for the eNOS, may contribute to it’s uncoupling, it could influence NO production in an additional manner (Berka et al. 2004). However, the role of urea cycle dysfunction was not investigated in this study and could be a proposal for further experiments.

### 4.2. FGF23 regulate aortic Cyp27b1 (1α-OHase) expression and superoxide production

The 1α-OHase is a member of the Cytochorome P450 monooxigenases, located on the inner mitochondrial membrane. Despite drug metabolism or the synthesis of cholesterol, steroids
and other lipids, the main activity of the enzyme is the conversion of 25-hydroxyvitamin D₃ into 1α-25-hydroxyvitamin D₃, the biological active form of vitamin D. The main counter player for the 1α-OHase is the 24-hydroxylase, which degrades active form of vitamin D. 1α-OHase provides its main activity in the proximal tubule cells of the kidney, where it maintains systemic vitamin D levels. It is generally accepted that FGF23 regulates renal vitamin D production through suppression of 1α-OHase expression and that bone FGF23 expression is regulated by vitamin D bone signalling in a classical feed-back loop regulation (Liu 2006).

However in extra-renal tissue the regulation of vitamin D expression and signalling is different as it is described in the kidney. Here, 1,25(OH)₂D₃ serves as an paracrine or autocrine factor, that initiate immune responses, cell growth, differentiation, and secretory function (Dusso et al. 2005), and does not contribute to the systemic serum levels. It is suggested that the 1α-OHase in extra-renal sites is mainly regulated from its own substrate (Daniel Zehnder et al. 2002a) or different inflammatory molecules (Daniel Zehnder et al. 2002b). Recent study show that the extra-renal regulation of Cyp27b1 is also triggered by FGF23, mainly through acting on the Erk1/2 signal pathway modulating the promoter activity of the Cyp27b1 gene (see Figure 8) (Chanakul et al. 2013). The authors suggested increased 1α-OHase expression in VSMC cells after recombinant FGF23 treatment for 21h.
In the present study we observed no regulatory effect of 100 ng/ml of recombinant FGF23 after 24 h incubation in aorta organ culture. Interestingly, combined recombinant FGF23 and inhibitor of superoxide production (Apocynin) treatment resulted in a $1\alpha$-OHase expression decrease, whereas treatment with Apocynin alone did not change the expression. Same result was also obtained for combined treatment of recombinant FGF23 and NO scavenger (N-Actetyl-Cystein). This data suggest that FGF23 suppressive effect on the aortic $1\alpha$-OHase expression supress local $1,25(\text{OH})_2\text{D}_3$ production when intracellular levels of NO and ROS in the cell are reduced (see Figure 8). In this way, higher systemic vitamin $\text{D}_3$ can transcriptionally activate eNOS (Andrukhova et al. 2014) which leads to the higher NO levels.

Figure 8: Suggested endothelial FGF23 signalling mechanism.

Graphical model of FGF23 regulatory pathway in the vascular tissue. Receptor binding of FGF23 is believed to activate Erk 1/2, a potential intracellular mediator which can induce superoxide (SO) by directly activating NADPH-oxidase. Increased ROS in the cell may activate vitamin D-driving tissue-specific antioxidative machinery which can directly trigger eNOS transcription. At the same time, Erk 1/2 known to inhibit $1\alpha$-OHase transcription and can phosphorylate eNOS, leading to its specific inactivation.
and protects endothelial cells by reducing ROS levels (Kanikarla Marie and Jain 2014). On the other hand reduction in the ROS level may activate the FGF23 action at supraphysiological concentrations (for example during CKD pathology) and decrease 1α-OHase expression leading to the decreased local vitamin D3 production.

It was previously reported that activation of the Erk1/2 due to hypoxia in the human pulmonary artery endothelial cells lead to the activation of NADPH-Oxidase (Parinandi et al. 2003). Thus, inhibition of the NADPH-Oxidase with Apocynin in our experiments could lead to a shift in the Erk 1/2 activity which may synergise the FGF23 mediated down regulation of Cyp27b1 (see Figure 8). However neither ERK1/2 nor superoxide and NO levels were investigated in this experiment and should be explored in the subsequent studies.

At the same time with the ability as a scavenger for NO, N-Acetyl-Cystein is described to be a potential cysteindonor, which can upregulate glutathione production, an antioxidative molecule (Rushworth and Megson 2014) in the endothelium (Berk 2008). Therefore, the effect of N-Acetyl-Cystein observed in our study could be due to the decreased ROS production and could recapitulate the Apocynin result.

Collectively, the data of this chapter suggested that FGF23 action on endothelial cells depends on the intracellular oxidative stress status. The study of others show that FGF23 elevates the superoxide levels in endothelial cells and reduces NO bioavailability (Silswal et al. 2014). This could be achieved, through a down-regulation of Cyp27b1, with a subsequent decrease of ROS-level which blunts 1,25(OH)2D3 availability resulting in the decrease of the NO production. However, it seems that this is only happening when the basal levels of superoxides are lower or antioxidative molecules such as glutathione are present in the cell, since recombinant FGF23 treatment alone does not change the Cyp27b1 expression. To further explore the mechanisms underlying these observations additional experiments should be performed to analyse 1α-OHase protein levels to conform the action of FGF23 on the endothelial oxidative status and to reveal the role of Erk 1/2 signalling in this mechanism.

4.3. Role of Klotho in FGF23-mediated signalling in the aorta

The role of Klotho in non-renal signalling of FGF23 remains controversial. Klotho as soluble factor was described to prevent aortic tissue calcifications (M. C. Hu et al. 2011). In Klotho
deficient mice circulating FGF23 level is elevated (Razzaque 2009). Klotho was defined as a co-receptor for FGF23 signalling in regulation of the phosphate in the proximal tubule cells and as a calcium and sodium in the distal tubular cells in the kidney (Andrukhova et al., 2014). However, in the aorta only very low or even no expression of Klotho was reported (Silswal et al. 2014), implicating that Klotho is not necessary for endothelial FGF23 signalling.

We observed the lowest relative expression values for eNOS (on mRNA and protein level) in Klotho−/−/VDRΔ/Δ compound mutant. Though, eNOS mRNA and protein expression level values were not significantly different from those in the VDRΔ/Δ mice indicating that the effect in eNOS expression change could mainly derive from the VDR deficiency. Interestingly, we were also not able to detect changes in aortic total nitrogen in these animals as compared to the wild type animals. In general the role of Klotho in FGF23-mediated signalling in the aorta remains rather elusive. However, the data of the present study suggested presence of the FGF23 signalling regulating eNOS and Cyp27b1 expressions in the aorta in a Klotho-independent fashion. Further work has to be done in order to verify the importance of the soluble Klotho in the endothelial NO and ROS production.
5. Deutsche Zusammenfassung der Bachelorarbeit

Das für den Mineralhaushalt von Vertebraten essentielle Vitamin D₃ (1α,25-Dihydroxyvitamin D₃) ist neben seiner klassischen Wirkung auf Niere und Dünndarm auch im Verdacht klinisch relevante, nicht klassische Wirkungen auf das kardiovaskuläre System auszuüben. Ein niedriger Vitaminspiegel wurde hierbei mit typischen Erkrankungen des Herzens (linksventrikuläre Hypertrophie) und der Gefäße (arterielle Kalzifizierung oder Endotheldysfunktion) in Verbindung gebracht. Eine vorhergehende Publikation konnte bereits eine direkte transkriptionelle Regulation von Vitamin D auf die endotheliale Stickstoffmonoxid-Synthase (eNOS), der Hauptquelle des vasoaktiven NO beweisen. Diese Bachelorarbeit soll an diesen Erkenntnissen anknüpfen und vor allem die Rolle des Fibroblasten Wachstumsfaktors 23 (FGF23), ein Hormon das von Osteozyten und –blasten produziert wird und für die Regulation des Phosphathaushaltes im Zusammenspiel mit Vitamin D wichtig ist, auf die Expression eNOS sowie die lokale Vitamin D Produktion in den Blutgefäßen behandeln. Dabei wurde schon ein Zusammenhang von vermindelter NO Bioverfügbarkeit durch Superoxiden in der Zelle und hohen Serumspiegeln von FGF23 verifiziert. Daher stellen wir die Hypothese, dass FGF23 in die endotheliale NO Produktion involviert ist, möglicherweise über die Regulation der lokalen Vitamin D Synthese und in Abhängigkeit des Ko-Rezeptors Klotho.

Für die Untersuchung unserer Hypothese haben wir Aorta Gewebe von verschiedenen, drei Monate alten Knockout Mäusen verwendet: mit verändertem Vitamin D Rezeptor (VDRΔ/Δ), VDR und FGF-23 (Fgf23 −/−/VDRΔ/Δ) sowie VDR und Klotho (Klotho −/−/VDRΔ/Δ), wobei Wildtyp Tiere als Referenz dienten. Die relative Gen- und Proteinexpression wurde mittels qPCR und Western Blot Analyse ermittelt. Hier konnten wir einen signifikanten (p<0,05) Anstieg in Fgf23 −/−/VDRΔ/Δ sowie einen signifikanten Abfall bei Klotho −/−/VDRΔ/Δ Tiere der Nos3 Genexpression beobachten.

Eine Messung der Gesamt-Stickstoffmonoxidkonzentration aus Gewebehomogenaten wurde als Indikator für die Bioverfügbarkeit von NO verwendet. Wir überprüften ob hohe FGF23 Spiegeln (in CKD Mäusen - ein Modell für chronische Niereninsuffizienz und auch in Klotho −/−/VDRΔ/Δ Mäusen) den Gesamt-Stickstoffmonoxidspiegel erniedrigen, konnten jedoch nur
ein leicht erniedrigter Wert für CKD Tiere im Vergleich zu operierten Kontrolltieren feststellen.

Eine Behandlung, von entnommenem Aorta Gewebe über 24 h, mit rekombinantem FGF23 zeigte nur dann eine Verminderung der 1α-OHase mRNA Expression, wenn zusätzlich ein Inhibitor der Superoxid-Produktion oder N-Acetyl-Cytein (ein NO-Fänger und antioxidatives Molekül) in der Kultur vorlag.

Zusammenfassend haben wir Indizien, dass FGF23 auf die Produktion von NO vermindert, möglicherweise über Verminderung der 1α-OHase Expression in einem Superoxid abhängigen Mechanismus.
6. Summary of the bachelor thesis

In this work we aimed to investigate if Fibroblast Growth Factor 23 (FGF23) has a potential effect on nitric oxide production, vitamin D3 (1α,25-dihydroxyvitamin D₃) metabolism or superoxide depended intracellular signalling in mouse aortic tissue. Recent human epidemiological studies show that low vitamin D serum levels correlates with arterial stiffness, left ventricular hypertrophy or endothelial dysfunction suggesting pleiotropic non-classical effect of vitamin D on the cardiovascular system. Studies from our group illustrate that vitamin D act is a direct transcriptional activator of the Nos3 (endothelial nitric oxide synthase). Additionally, it was shown that elevated FGF23 levels increase superoxide and NO production in endothelium and transcriptionally regulates the renal vitamin D production via 1α-hydroxylase. Therefore, we hypothesise that FGF23 could be involved in endothelial NO production by possible regulation of the local aortic vitamin D production and that this signalling requires FGF23 co-receptor molecule Klotho.

In our experiments we examined aorta thoracalis of three month-old male C57BL/6 wild type mice, vitamin D receptor deficient (VDRΔΔ) mutants, and animals lacking FGF23 or Klotho together with VDR ablation (Fgf23⁻/⁻/VDRΔΔ, Klotho⁻/⁻/VDRΔΔ). Relative eNOS mRNA and protein expression levels were determined in all experimental animal groups using qRT-PCR and Western blot analysis. We found significantly increased (p<0.05) aortic eNOS mRNA expression but not protein expression in Fgf23⁻/⁻/VDRΔΔ compound mutants as compared to the other animal groups. In Klotho⁻/⁻/VDRΔΔ compound mutants aortic eNOS expression was decreased at both mRNA and protein levels.

To analyse if total NO production in the aortic tissue could be regulated by excessive FGF23 level we further measured total nitric oxide content in the aortic homogenates of mice undergoing chronic kidney disease and in Klotho⁻/⁻/VDRΔΔ compound mutants using ozone – chemoluminescence based technology (NO Analyser 280i Sievers, Boulder, USA). Both, CKD and Klotho⁻/⁻/VDRΔΔ compound mutants were shown to have elevated circulating FGF23 level. Here we found unchanged total nitric oxide content for Klotho⁻/⁻/VDRΔΔ mutants compared to wild type and slightly decreased total nitrogen content in CKD animals as compared to the sham operated animals.
Recombinant FGF23 treatment of aortic organ culture for 24h did not change 1-αOHase mRNA expression when treated alone, whereas FGF23 co-treatment with the NO scavenger or inhibitor of superoxide production significantly decreased aortic 1-αOHase mRNA expression.

Collectively, the data of the present study provide clear evidence that FGF23 signalling can modulate endothelial eNOS expression and decrease NO bioavailability possibly through superoxide dependent regulation of 1-αOHase mediated local vitamin D production.
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