Signal transduction involving GSK3 and activation of CASR by magnesium during medial vascular calcification

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Summary

Medial vascular calcification, the deposition of calcium-phosphate in the media of the vasculature, is considered as an active and highly regulated process. This is believed to be controlled by osteo/chondrogenic transformation of vascular smooth muscle cells. Until now, the underlying mechanisms are incompletely understood. The calcification of arterial tissue is closely associated with cardiovascular mortality, but due to the incomplete understanding about the mechanisms of vascular calcification, no therapeutic treatment exists. This work studied new signaling pathways in vascular smooth muscle cell calcification.

A putative therapeutic strategy may be magnesium supplementation, which counteracts vascular calcification. Therefore, possible signaling pathways to mediate the effects of magnesium were investigated in vascular smooth muscle cells. The phosphate-induced calcification and the mRNA expression of the osteogenic markers core-binding factor α1, msh homeobox 2, and tissue-nonspecific alkaline phosphatase in human aortic smooth muscle cells were ameliorated by additional treatment with magnesium.

The presence of magnesium chloride enhanced mRNA expression of the calcium-sensing receptor in human aortic smooth muscle cells. Most importantly, the blunting effects of magnesium on calcification and osteogenic markers mRNA expression were reproduced by the calcium-sensing receptors activator gadolinium. Furthermore, the anti-calcific effects of Magnesium were abolished by silencing of the calcium-sensing receptor or addition of its antagonist NPS-2143. Furthermore, cholecalciferol overload in mice led to vascular calcification and upregulated aortic osteogenic markers core-binding factor α1, msh homeobox 2, tissue-nonspecific alkaline phosphatase and collagen type I, collagen type III and fibronectin mRNA expression in mice. These effects were ameliorated by supplementary treatment with magnesium chloride.

Further studies investigated a contribution of the Glycogen synthase kinase 3 (GSK-3) during vascular calcification. The calcification and upregulated expression of osteogenic markers msh homeobox 2, core-binding factor α1 and tissue-nonspecific alkaline

phosphatase as well as mRNA expression of type III sodium-dependent phosphate transporter and plasminogen activator inhibitor 1 following cholecalciferol overload in mice was blunted in Gsk-3α/β double knock-in mice, which lack the functional AKT/SGK phosphorylation sites. In addition, phosphate exposure induced calcification and osteogenic markers expression in aortic ring explants from GSK-3 wild type mice, effects blunted in aortic ring explants from GSK-3 knock-in mice.

The current observations thus shed new light on the signaling pathways of vascular smooth muscle cell calcification. The anti-calcific effects of magnesium are at least in part mediated via the calcium-sensing receptor. Furthermore, the GSK3 is apparently involved in the pro-calcifying signaling cascades. These observations extend the current knowledge on the mechanisms of calcification, an understanding that may ultimately lead to development of a therapeutic approach.

Zusammenfassung

Mediale Gefäßverkalkung, die Ablagerung von Kalzium-Phosphat in den medialen Schichten der Arterien, wird als ein aktiver und hochgradig regulierter Prozess angesehen. Dieser wird wahrscheinlich durch osteo-/chondrogene Transdifferenzierung von glatten Gefäßmuskelzellen kontrolliert. Bislang sind die zugrundeliegenden Signalwege nicht genau bekannt. Die Gefäßverkalkung ist stark assoziiert mit kardiovaskulärer Mortalität, aufgrund des fehlenden Wissens über die zugrundeliegenden Signalwege gibt es bislang keine therapeutische Behandlung. Diese Arbeit untersuchte daher neue Signalwege der Verkalkung von Gefäßmuskelzellen

Eine mögliche Behandlungsstrategie könnte Magnesiumsupplementation darstellen, welche die Gefäßverkalkung hemmen könnte. Es wurden daher Signalwege der protektiven Wirkung von Magnesium untersucht. Die Phosphat-induzierte Verkalkung und mRNA Expression der osteogenen Marker core-binding factor α1, msh homeobox 2, und tissue-nonspecific alkaline phosphatase wurde durch Magnesium abgeschwächt. Magnesium erhöhte die mRNA Expression des calcium-sensing recptors in humanen Gefäßmuskelzellen. Der Effekt von Magnesium auf die phosphat-induzierte Verkalkung und Expression der osteogenen Marker wurden durch den calcium-sensing receptor Agonisten Gadolinium repliziert. Silencing des calcium-sensing receptors oder der Antagonist NPS-2143 blockierten die anti-kalzifizierende Wirkung von Magnesium. Cholecalciferol-Überladung in Mäusen induzierte in Gefäßen Verkalkung und vermehrte mRNA Expression der osteogenen Marker core-binding factor α1, msh homeobox 2, tissue-nonspecific alkaline phosphatase, sowie collagen type I, collagen type III und fibronectin. Diese Effekte wurden durch Magnesium abgeschwächt.

Weitere Versuche untersuchten eine Beteiligung der Glycogen synthase kinase 3 (GSK-3) während vaskulärer Verkalkung. Die Verkalkung und Erhöhung der osteogenen Marker msh homeobox 2, core-binding factor α 1 und tissue-nonspecific alkaline phosphatase, sowie mRNA Expression des type III sodium-dependent phosphate transporter und plasminogen activator inhibitor 1 nach Cholecalciferol-Überladung waren abgeschwächt in Gsk-3α/β Doppel-knock-in Mäusen, in denen eine GSK3 Phosphorylierungsstelle für

AKT/SGK fehlt. Ebenso war die Verkalkung und Expression der osteogenen Marker in Phosphat-exponierten Gefäßringen von GSK3-knock-in Mäusen abgeschwächt.

Diese Beobachtungen liefern damit neue Erkenntnisse über die Signalwege der Verkalkung von glatten Gefäßmuskelzellen. Die anti-kalzifizierenden Effekte von Magnesium wurden zumindest zum Teil durch den calcium-sensing receptor vermittelt. Ebenso ist die GSK3 anscheinend in die Signalwege der Gefäßverkalkung involviert. Diese Ergebnisse erweitern das Wissen um die Mechanismen der Gefäßverkalkung, das ultimativ dazu beitragen könnte, eine therapeutische Strategie zur Verhinderung der Verkalkung zu entwickeln.

Abbreviations

ALPL	tissue-nonspecific alkaline phosphatase
CASR	calcium-sensing receptor
CBFA1	core-binding factor a 1
CKD	chronic kidney disease
Col1a1	collagen type I
Col3a1	collagen type III
CTR	control
Fbn	fibronectin
FGF23	fibroblast growth factor 23
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
$GdCl_3$	gadolinium trichloride
HAoSMCs	human aortic smooth muscle cells
MSX2	msh homeobox 2
Pi	phosphate
PTH	parathyroid hormone
vitD	vitamin D
VSMCs	vascular smooth muscle cells
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
Gsk-3	glycogen synthase kinase 3
Msx2	msh homeobox 2
Pai1	plasminogen activator inhibitor-1
Pi	phosphate

Pit1	type III sodium-dependent phosphate transporter
AKT	protein kinase B
Sgk1	serum- and glucocorticoid-inducible kinase 1
FBS	fetal bovine serum
IU	international units
Hap	hydroxyapatite
ECL	enhanced chemiluminescence
siRNA	small interfering RNA
DMEM	Dulbecco’s modified eagle’s medium
SDS	sodium dodecyl sulfate
PBS	phosphate-buffered saline
HRP	horseradish peroxidase
PVDF	polyvinylidene difluoride membrane
TRPM	transient receptor potential melastatin

1. Introduction

1.1 Chronic kidney disease

1.1.1 Definition of chronic kidney disease (CKD)

According to the (KDIGO) Kidney Disease Improving Global Outcomes, chronic kidney disease (CKD) is a global public health burden, associated with severe consequences of end-stage renal failure, increased risk for cardiovascular disease (CVD), and death.[1, 2] It is classified into 6 stages depending on the patient's status of glomerular filtration rate (GFR), as an evaluation of filtration capability of the kidneys.[1, 3] Stage-1 has regular filtration capability of the kidney but is related to those persons who are at danger of developing kidney failure, especially patients suffering from proteinuria or hematuria.[3, 4] Stage-2 is considered in levels of a GFR from 60 to 90 mL per minute.[3, 4] Stage-3 is considered with a glomerular filtration rate of 30 to 60 mL per minute.[1, 3, 4] Stage-4 is considered as a glomerular filtration rate of 15 to 30 mL per minute.[1, 3, 4] Stage-5 is considered a glomerular filtration rate of less than 15 mL per minute but still not on dialysis.[1, 5] The final result of a failing kidney is the stage of patients who have a glomerular filtration rate of less than 15 mL per minute and need dialysis to supplement for the their lost kidney function.[1-3, 6]

1.1.2 How does CKD develop

Chronic kidney disease occurs as lack of kidney filtration capability that develops over extended sequence of time.[2, 7, 8] The chronic loss of kidney filtration capability could be related to prior occurrences of acute kidney disease with following long term renal injury, or could be related to diseases that led to advanced weakening of the kidneys, such as primary renal diseases (glomerulonephritis) or secondary injury due to diseases like diabetes, hypertension, or auto-immune syndromes.[9, 10] The shortfall of renal filtration capability worsens over time, but through medical care for the underlying disease, this impairment could be resolved and in many situations, the renal filtration capability might improve.[3, 9, 11] On the other side, several patients with chronic kidney disease, particularly when develop Stage 4 or Stage 5 will reach the level of end stage

renal disease (ESRD) which is the state of requiring dialysis or kidney transplantation.[1, 12] Gradual continuing damage of renal filtration capability over the time of years leads therefore to chronic kidney disease.[2, 5, 13] A huge effort is being made to attempt halting the progress of chronic kidney disease.[12, 14] Chronic kidney disease develops without drastic symptoms, and unfortunately, the capability to diagnose early chronic kidney disease is paramount to stop the decline of renal function.[12, 14, 15] Patients with terminal renal disease require dialysis or transplantation of a kidney to cover the impairment caused by the lack of renal filtration.[16]

1.1.3 How can CKD be treated

- Pharmacological management:

Optimal pharmacological management of chronic kidney disease (CKD) may be able to reduce its advance to end-stage renal disease (ESRD).[17] Nevertheless, the benefits and exact treatment regimens of medical treatments are not clearly defined.[17, 18] Blood pressure managing via angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers (ARBs) are considered a cornerstone for CKD therapy.[16-18] Glycemic managing in diabetes could be helpful for a delayed development of CKD.[16, 17, 19] Different metabolic disorders associated with CKD might be useful as treatment goals, such as metabolic acidosis, hyperphosphatemia, and vitamin D deficiency. [16, 17, 20]

CKD could also be associated with hypercholesteremia and hyperlipidemia, so the need for statins to reduce body cholesterol concentrations is a high important step in CKD treatment.[16, 17, 21] However, the known benefits of statins in patients without CKD may be impaired in the setting of end stage renal disease .[22] CKD patients typically also develop anemia, and require substitution of erythropoietin, iron and eventually even transfusions. [16, 17, 21] By fluid retention or volume overload, diuretics could offer the best assistance to reduce this sodium and water retention and overload.[16, 17, 21]

In CKD, the concentrations of vitamin D (which is important for calcium resorption) are decreased and phosphate levels will rise, which leads to reduced levels of calcium.[17, 21] Subsequently, hyperparathyroidism develops. [17, 21] Currently, phosphate binders

are the most widely used clinical approach to control phosphate and (as a result) calcium levels in the blood.[21, 23]

- Dietary management:

  Dietary interventions may also be useful, as a reduced protein content in the diet may be beneficial in CKD, but is still controversial.[21, 24] In patients with CKD, also increased concentrations of potassium (hyperkalemia) could be a life threatening complication.[21, 23, 25] As hyperkaliemia may induce irregularity of heart functions and activity, a diet that prevents high intake of potassium may be required in later stages of CKD.[17, 23]

- End-Stage Renal Disease Treatment:

  The available treatment options for end-state CKD remain dialysis and renal transplant, and since both could have serious risks for the patients survival, physicians try to delay these choices as long as possible.[25]

  Dialysis is a technique for filtering end products and extra liquids from blood.[12, 25] There are numerous types of dialysis available to replace physiological renal function.[2, 25] The two major kinds of dialysis are hemodialysis and peritoneal dialysis.[23, 25] In hemodialysis, the blood is filtered in a dialysis machine outside of the body.[23, 25] In peritoneal dialysis, the abdominal cavity is filled with a dialysis solution via a catheter.[26-28] The solution will absorb waste products, and this solution can be then removed from the body.[26, 27] As dialysis is usually performed three times a week, it implicates a major strain on the patients quality of life.[26] Dialysis could also carry some risk of complications, such as infections.[26, 27]

  Kidney transplant can be sometimes more suitable than dialysis, but is still associated with complications.[26, 27, 29] A living-kidney donation from a sibling or other close relative is considered the best choice for kidney transplant.[27, 29] Alternatively, kidney transplants are also obtainable from a deceased donor.[26, 27, 29] However, renal transplants may lead to a large risk of infection as it will require lifelong immunosuppression.[29, 30]

1.1.4 CKD-associated complications

Chronic kidney disease is associated with critical complications, that also determine the clinical course of the disease.

- Volume overload:

In chronic kidney disease, water and sodium retention may occur.[29, 31] The inability to eliminate enough salts and fluids could lead to volume overload.[29, 31] This retention is frequently observed when the GFR drops to fewer than 10-15mL/min.[29, 31]

- Hypertension:

Hypertension is closely associated with chronic kidney disease. Over 80% of chronic kidney disease patients are suffering from increased blood pressure.[29, 31] Hypertension is usually a consequence of volume excess and sodium retention, due to the loss of the renal capability to eliminate extra water and sodium.[29, 31, 32] Furthermore, the renin-angiotensin-aldosterone system, that inherits an important function in controlling blood pressure may be overactivated in chronic kidney disease.[31, 32] While hypertension could lead to chronic kidney disease, suitable management of blood pressure and inhibition of the renin-angiotensin-aldosterone system is considered an essential approach to slow CKD-progression and reduced cardiovascular complications.[31-33]

- Hyperkalemia:

Hyperkalemia could develop in CKD patients, due to the decreased capability to eliminate potassium through the kidney, mostly in patients with GFR less than 20-25mL/min.[29, 32] High potassium concentrations could also be observed in the initial phases of chronic kidney disease, mainly in patients whose diet is potassium-rich or those with decreased aldosterone concentrations.[31, 32]

Precautionary actions could be advised to patients with CKD to prevent increased potassium levels.[31, 32] Beginning of a low potassium levels diet, with limit between 1500-2700 mg/day, could decrease the absorption of the existing potassium.[31, 32] Stopping treatments that potentially raise potassium concentrations could also be

beneficial.[34] Potassium concentrations have to be carefully supervised and controlled in CKD patients to avoid the danger of hyperkalemia.[34]

- Dyslipidemia:

  A dysregulation of lipid metabolism could occur in chronic kidney disease patients, mainly observed as increased levels of triglyceride.[34] While hypertriglyceridemia could possibly exacerbate cardiovascular diseases and promote atherosclerosis, the therapeutic options are currently suffering from a lack of clinical data in the CKD population.[34, 35]

- Metabolic acidosis:

  Metabolic acidosis could develop in end-stage chronic kidney disease, and usually becomes symptomatic with muscle deterioration and reduction of the lean body mass.[36] Chronic kidney disease could lead to a reduced excretion of ammonia in the proximal tubules,[36, 37] which could cause higher acid concentrations in the blood.[36] Furthermore, accumulation of phosphates, sulfates, and organic anions could lead also to high acid concentrations.[36] This metabolic acidosis induces a negative influence on protein formation and distribution, which could be involved in the effects of muscle fatigue, and reduction of lean body mass.[36] While bone tissue could serve as protection form increased blood acid concentrations, metabolic acidosis could unfortunately also cause bone mineralization defects .[36, 38]

- Anemia:

  Dysregulation of hematopoiesis develops early in chronic kidney disease, and progresses in severity as the renal disease progresses, influencing around 8% of CKD stage 1 patients and almost 53% of CKD Stage 5 patients.[36, 38] Reduced kidney secretion of erythropoietin in CKD patients impairs the stimulus to the bone marrow to generate red blood cells, causing decreased oxygen-holding capability of the blood and decreased red blood cell persistence.[38, 39] Uremia and the accumulation of uremic toxins, unwanted molecules in the blood, could also lead to the loss of platelet activity.[38]

However, the presumably most severe complication of CKD has been termed mineral-bone disorder. Mineral-bone disorder is a common complication of CKD, because of the disturbed stability of mineral concentrations in the body, and is usually recognized as a syndrome: chronic kidney disease - mineral-bone disorder (CKD-MBD) syndrome.[40]

1.2 Mineral-bone disorder

1.2.1 Definition of mineral-bone disorder

Mineral-bone disorder in CKD develops due to the failing kidneys and irregular hormone concentrations, as well as imbalance the concentrations of calcium and phosphate in patient's blood.[36, 41] Bone and mineral disorder mainly occurs in people with CKD and especially to the majority of patients undergoing dialysis.[36]

Earlier, mineral-bone disorder was called "renal osteodystrophy" to define mineral and hormonal imbalance as a result for CKD.[36, 41] Nowadays, it has been recognized, that also other organs and tissues are involved in the detrimental dysregulation of mineral balance and endocrine regulation CKD.[40, 42] Thus, CKD-MBD is a syndrome that not only affects the bones, but also heart, and blood vessels.[40]

1.2.2 How does CKD-MBD develop

Under physiological conditions, phosphate and calcium concentrations are strictly controlled and delicately stable in the body.[40] Calcium and phosphate in the diet are absorbed in the small intestine, and are usually filtrated and re-absorbed by the kidneys.[42] Phosphate binds with calcium producing a hydroxyapatite-complex to be stored in the bones as bone minerals.[42] To keep the balance of calcium and phosphate, the parathyroid hormone (PTH) functions to decrease blood phosphate concentrations and raise blood calcium concentrations.[42, 43] Similarly, the phosphaturic hormone fibroblast growth factor 23 (FGF23) induces phosphate loss through the kidney, and may serve as a long term phosphate regulator .[44]

The active form of vitamin D, which is known as calcitriol, is involved in maintaining phosphate and calcium concentrations in the blood, by promoting intestinal reabsorption

of calcium and phosphate.[45] CKD patients typically display elevated phosphate levels due to impaired phosphate excretion, reduced calcium levels, increased production of parathyroid hormone and FGF23 and reduced levels of calcitriol.[45] These alterations are associated with detrimental sequalae.[46]

1.2.3 Consequences of CKD-MBD

The most typical complications of mineral and bone disorder in CKD are disturbed bone mineralization and cardiovascular disease due to ectopic calcifications.[46]

Impaired bone mineralization develops due to long-term hyperphosphatemia and hyperparathyroidism. [46] The resulting bone mineralization in turn promotes hyperphosphatemia and further exacerbation of a deranged endocrine system.[46, 47] The hyperphosphatemia of mineral and bone disorder in CKD can lead to vascular calcifications, a determinant of patients mortality due to its effects on cardiovascular events.[47]

Thus, when GFR drops to less than 20-25 mL/min, the kidneys are unable to maintain the sufficient excretion of phosphate, which leads to hyperphosphatemia.[47, 48] That activates the release of parathyroid hormone and FGF23.[48] As an outcome, vitamin D concentrations are decreased.[48] This leads to a reduction in calcium uptake through both small intestine and bones, and calcium precipitates with phosphate, resulting in reduced blood calcium concentrations.[48, 49] This vicious cycle sets the stage for extraosseous calcifications, a hallmark of CKD-MBD.

1.2.4 Mineral and bone disorder in chronic kidney disease treatment

Up until now, the treatment options for CKD-NBD are insufficient: taking care of mineral and bone disorder in CKD involves managing parathyroid hormone concentrations through pharmacological agents, changing diet habits, and finally by dialysis.[48] When these options fail to correct parathyroid hormone concentrations in the blood, ultimately a parathyroidectomy may be performed .[48, 50]

- Dietary modification, and nutrition:

Adapting the diet could improve mineral and bone disorder in CKD.[50] Decreasing dietary phosphate is considered essential to reduce phosphate load.[51] Mainly all types of food contain phosphate, but the phosphate load is especially high in processed and packaged food.[50] Food manufacturers rely on phosphates as preservatives to delay their expiry dates and other factors relevant for the food industry.[50] Patients with CKD, especially in end-stage renal disease are advised to reduce consumption of processed and packaged food.[50] Many beverages and other types of foods also include increased concentrations of phosphate, especially beer, cheese, cocoa, dark sodas, dried beans, milk, nuts, peanut butter and peas which should be avoided by CKD patients.[51]

- Pharmacological agents and supplements:

Various pharmacological agents have been considered to contribute to protection of the bones.[52] In situations of insufficient levels of calcitriol, calcitriol may be supplemented.[52] Calcitriol reduces parathyroid hormone concentrations.[50, 51] Furthermore, calcium supplementations may be used.[48, 53]

Cinacalcet is a calcium-sensing receptor activator that is able to reduce parathyroid hormone concentrations by exerting an effect as calcium on the parathyroid glands. [51, 53] Mostly, this treatment is only used with dialysis patients.[51]

In addition, oral phosphate binders like calcium carbonate, calcium acetate , or lanthanum carbonate, can be used with diets and different types of food to bind phosphate in the bowel.[51] These agents reduce the uptake of phosphate in the body.[51, 54]

The already mentioned variations in mineral metabolism are accompanied with cardiovascular complications and mortality in CKD.[51] Most important, hyperphosphatemia is a key trigger for vascular calcification.[48, 55]

1.3 Medial vascular calcification

1.3.1 Definition of vascular calcification

Medial vascular calcification is defined as the imbalanced pathological distribution of minerals in form of calcium phosphate complexes in the vascular tissues.[56] This could occur during the aging process, but it is strongly augmented in CKD, diabetes mellitus and specific genetic diseases.[56] The most potent trigger of vascular calcification is chronic kidney disease (CKD).[56, 57] Vascular calcification was considered to be typical of in end-stage renal disease (ESRD) and with patients depending on dialysis, but the recent studies demonstrated that vascular calcification could already start earlier, and could develop even in the second stage of CKD.[56] This inappropriate calcification in the vessel wall leads to increased arterial stiffness, high pulse pressure, and left ventricular hypertrophy.[56, 58] Altogether, this presumably promotes plaque instability and impaired organ perfusion.[58]

1.3.2 Development of vascular calcification

Vascular calcification can mostly occur in two sites of the vascular walls, the intima and the media.[58] The intimal layer of the artery consists of endothelial cells covered by a external layer of elastic fibers.[58] Calcification at this side is associated with atherosclerosis due to dyslipidemia, and both inflammation and thickening of the intimal layer contribute to this process.[58, 59] This leads to formation of plaques on the inner vascular wall with luminal obstruction.[58]

The medial vascular layer consists of smooth muscle cells embedded in connective tissue, which is usually made of elastin. [59] During medial calcification, this layer develops extensive mineralization [59]. Although patients with CKD are at increased risk to develop both kinds of vascular calcification, the medial calcification is most prominent during CKD.[60] This calcification is associated with hyperphosphatemia and strongly associated with cardiovascular mortality of the patients.[61] Especially medial calcification has therefore originated as a critical consequence of CKD, but the underlying mechanisms are incompletely understood.

1.3.3 Risk factors for Vascular calcification

In CKD, multiple traditional such as hypertension and diabetes, but also non-traditional risk factors, most importantly hyperphosphatemia may promote medial vascular calcification:

- Hypertension has been associated with vascular vulnerability and calcification.[61] The renin-angiotensin system is a key pathogenic factor in VSMC programmed cell growth, differentiation and death, which is involved in the development of vascular calcification.[61, 62]
- Hyperphosphatemia is the abnormal increase of serum phosphate concentrations, typically occurring in late stage CKD. [61-63] Hyperphosphatemia typically remains asymptomatic, but is considered a most powerful stimulus for vascular calcification. [63-65]
- Diabetic patients have higher risk to develop vascular calcification as compared to non-diabetic people.[62] The underlying mechanisms are not clearly defined, but may involve increased inflammation in the vascular wall.[64, 65]

1.3.4 Treatment strategy of Vascular calcification

Due to the lack of knowledge about the mechanisms of medial vascular calcification, there are no therapeutic concepts that can be widely used in clinical routine.[64] Currently, the main approach in CKD is to reduce hyperphosphatemia by the use of phosphate binders.[66, 67] Other therapeutic concepts are still experimental and cannot be applied to the general CKD population.[64, 66] In order to find more specific treatments for vascular calcification, both the biology and chemistry of vascular calcification should be meticulously understood.[68]

Recently, it was recognized that vascular calcification is not a passive phenomenon, but it involves dynamic remodeling of vascular smooth muscle cells into different phenotypes.[69] Interfering in these active processes may be the most promising strategy to develop an intervention to target vascular calcification.[70]

1.4 Vascular smooth muscle cells

Vascular smooth muscle cells (VSMCs) are non-striated contractile cells, found in the medial layer of the arteries.[71] They are cellular constituents of the blood vessels walls that are involved in basic flexibility and control the vessel's diameter by relaxing and contracting actively as a reaction to the vasoactive stimulation.[71] The distinguished status of the VSMC is categorized by special contractile proteins, ion channels, and cell surface receptors that control the contractile procedure in the cells.[71, 72] Furthermore, as a reaction to injury, VSMCs are able to change the production of extracellular matrix proteins, and are able to migrate and proliferate.[72] Vascular smooth muscles are considered as key regulators of vascular calcification.[71, 72]

1.4.1 Role of smooth muscle cells in vascular calcification

Calcification has been recognized as an active process, which is in large part regulated by phenotypical change of vascular smooth muscle cells (VSMC) in the vascular wall.[72] It has been decisively shown that VSMCs are capable to undergo transition of their phenotype into a calcific phenotype, that promotes vascular mineralization.[72]

Although the mechanisms and signaling pathways that lead to a calcific VSMC differentiation are not fully clarified, it is clear that complex pathways regulate this process, which differs from intimal atherosclerosis.[73] This includes differing risk factors and special triggers for VSMC phenotype differentiation and calcification.[73] It is also theorized that vascular calcification in CKD could be considered as the result of prematurely aging process of VSMCs.[74, 75]

1.4.2 Interference with vascular calcification

As discussed, no feasible treatment options exist for medial vascular calcification. It is considered, that optimal management of traditional risk factors may be beneficial.[76, 77] At least for intimal calcification, such benefits were shown.[77, 78] However, patients with chronic kidney disease exhibit non-traditional risk factors, such as uremia and

hyperphosphatemia, creating a unique environment for vascular calcification.[79, 80] Therefore, further therapeutic options are an important medical need.[81]

However, the underlying signaling pathways that promote vascular calcification are not completely understood. This understanding would be paramount to develop treatment strategies to prevent vascular calcification in CKD. Nonetheless, an important factor emerged, that may provide benefits in CKD: Magnesium has been considered to have beneficial effects on vascular calcification in CKD. [82, 83]

Magnesium supplementation has been considered to be a safe treatment in chronic kidney disease patients, and may actually interfere with vascular calcification.[83, 84] However, the mechanisms of Magnesium on VSMCS are not clearly defined.

Especially the intracellular signaling pathways, that mediate the phenotypical change of VSMCs are elusive. It was previously hypothesized, that the Phosphoinositide 3-kinase pathway may be involved in the signaling of VSMC-calcification, and that this effect could involve the Glykogensynthase-Kinase 3 (GSK3).[85, 86] GSK-3 is a cytoplasmic serine/threonine protein kinase that inhibits the glycogen synthase from glucose by its phosphorylation.[87] GSK-3 controls multiple cellular effects by modulating different signaling pathways which are necessary for cellular proliferation, cellular apoptosis, and stem cells renewal.[88, 89] As a result of the influence of GSK-3, it's hypothesized that the abnormal function of GSK-3 could be involved in different pathogenetic human diseases, and this could also extend to vascular calcification.[90] The GSK-3 effect could be invested to the possibility of new drug researches by influencing either GSK-3 inhibitors or activators.[90, 91]

2. Aim of the study

Vascular calcification is an active process promoted by osteo-/chondrogenic transformation of vascular smooth muscle cells (VSMCs).

Recently it was suggested, that magnesium may play a central role in vascular calcification, but the mechanism of action of magnesium is unclear. Therefore, possible signaling pathways modulated by magnesium were investigated.

Furthermore, it was hypothesized that gsk-3 phosphorylation has a possible role in the vascular calcification process. It was therefore investigated, whether regulation of GSK-3 by protein kinase B (AKT) and serum- and glucocorticoid-inducible kinase 1 (SGK1) are involved in the intracellular signaling of vascular calcification.

3. Materials and methods

- Cell culture of human aortic smooth muscle cells:

In order to investigate the effects of Magnesium on vascular smooth muscle cells during calcifying conditions, cell culture experiments were performed. For this purpose, commercially obtained primary human aortic smooth muscle cells (HAoSMCs; Life Technologies, Carlsbad, California, USA) were used. These cells were cultured n Waymouth's MB 752/1 medium and Ham's F-12 nutrient mixture (1: 1, Gibco, Life Technologies) according to standard cell culture techniques.

The medium and the nutrient culture of the human aortic smooth muscle cells, were supplemented with 100mg/ml streptomycin (Gibco, Life Technologies), 10% FBS (Gibco, Life Technologies) and 100 IU/ml penicillin. The cells were grown in a standard cell culture incubator with 5% carbon dioxide and utilized in the experiment's passages from passage 4 till passage 10.

To investigate the effects of the calcium-sensing receptor mediating possible effects of magnesium, silencing experiments were performed. These experiments were performed by transfection of human aortic smooth muscle cells with 10 nmol/l negative control siRNA (ID no. 4390843, Ambion, Life Technologies) or with 10 nmol/l CASR siRNA (ID no. s2440, Ambion, Life Technologies) using the siPORT amine transfection agent (Ambion, Life Technologies). Experiments were performed according to the manufacturer's protocol. After silencing for complete two days this silencing efficiency was shown by quantitative (RT-PCR) real time polymerase chain reaction.

To investigate calcifying conditions, human aortic smooth muscle cells were treated for the specified timespan with 5 mg/ml hydroxyapatite (<200nm particle size, Sigma-Aldrich), 2 mmol/l b-glycerophosphate (Sigma-Aldrich, St Louis, Missouri, USA), the specified concentrations of magnesium chloride respectively (0.5–2.5 mmol/l; Sigma-Aldrich), 50mmol/l GdCl3 (Sigma-Aldrich), or 100 nmol/l of the calcium-sensing receptor antagonist NPS-2143 (Sigma-Aldrich, stock dissolved in dimethyl sulfoxide). The identical same quantities of vehicle were used as control. Also, as a calcification medium in order

to cause calcium accumulation for calcification measurements, treatment for two weeks with 3 mmol/l sodium phosphate buffer (Sigma-Aldrich) was performed.

For long-term treatments, control HAoSMCs were grown in complete medium, which was replaced with fresh medium every two-three days at the same time with the replacement of complete medium supplemented with agents for the treated HAoSMCs groups.

- Animal experiments:

  In order to investigate the effects of magnesium and GSK3 on calcification in-vivo, animal experiments were performed. To induce calcification in mice, an established model of cholecalciferol overload was used. The animal experiments were performed according to the German legislation for the well-being of animals and all these experiments were approved by the local authorities.

  Induction of calcification was conducted by applying an overload of cholecalciferol by subcutaneous injection. C57BL/6 mice were given every day subcutaneous injections of cholecalciferol (400 000 IU/kg body weight) for three sequential days. The animal received a reduced calcium diet in pellet form (C1031, Altromin, Lage, Germany) and the drinking water was given as distilled water either with or without containing 8.3 g/l magnesium chloride (Sigma-Aldrich). Diet and drinking solutions were provided ad libitum.

  After six days the mice were sacrificed by cervical dislocation in isoflurane anesthesia. Blood was collected at time of sacrifice by retro-orbital puncture with heparinized capillaries. The murine tissues were collected, rapidly cleaned and immediately flash-frozen in liquid nitrogen. After collecting the blood samples from the mice, the samples were centrifuged and stored for further measurements.

  For measuring the concentrations of calcium and phosphate content, a photometric technique was utilized (FUJI FDC 3500i, Sysmex, Norderstedt, Germany). To measure Fibroblast growth factor 23 (FGF23) C-term (Immutopics, San Clemente, California, USA) an enzyme-linked immunosorbent assay (ELISA) was performed according to the protocol of the manufacturer.

Similar animal experiments were also performed to study the relevance of GSK-3 during vascular calcification. The experiments were conducted in the double knock-in GSK-3α/β21A/21A/9A/9A mice, which lack the effective AKT/SGK phosphorylation sites (gsk-3 knock-in) and the corresponding wild-type mice (gsk-3 wild type).[92, 93]These mice were injected with 400000 IU/kg body weight of cholecalciferol (Sigma-Aldrich) or control injection daily over three days. After six days mice were anesthetized by isoflurane and sacrificed, blood was collected by retro-orbital puncture, and tissues were collected and flash frozen in liquid nitrogen. To measure the concentrations of calcium and phosphorus, photometric measurements were applied (FUJI FDC 3500i, Sysmex, Norderstedt, Germany).

- Aortic ring explants:
  To investigate direct effects of GSK3 in the vasculature, experiments were conducted in aortic ring explants for ex-vivo treatments. From both gsk-3 knock-in and the corresponding gsk-3 wild type mice the aortas were extracted in a sterile manner. The endothelium was carefully removed and the vessel was cut into rings of 2mm to 3mm length. The rings were immediately cultured in DMEM medium (Thermo Fisher Scientific), 100 µg/ml streptomycin (Thermo Fisher Scientific) and 100 U/ml penicillin. The explants were treated for one week with 1.6 mM/l sodium phosphate buffer (Sigma-Aldrich) to induce calcifying conditions. Fresh media with the agents were added every two to three days.

- Calcium content assay:
  To evaluate the extent of calcification in the aortic tissues, these tissues were incubated in 0.6 mol/l HCl at 37 Celsius grades overnight. The obtained extract was then centrifuged and the calcium content in the supernatant from HCL was measured by a colorimetrical method, utilizing a calcium assay kit (BioAssay Systems, Hayward, California, USA) following the manufacturer’s instructions. The tissues were lysed in 0.1 mol/l NaOH/0.1% SDS and by using Bradford assay (Bio-Rad Laboratories, Hercules, California, USA) the total protein levels were determined. Calcium content was normalized according to the concentration of the protein.

To determine calcification of human aortic smooth muscle cells after induction of calcification, human aortic smooth muscle cells were decalcified over the night in 0.6 mol/l HCl solution. By utilizing the QuantiChrom Calcium assay kit (BioAssay Systems) following the manufacturer's instructions, the calcium content was measured in the colorimetrical kit. Furthermore, the human aortic smooth muscle cells were lysed in 0.1 mol/l NaOH/0.1% SDS. By utilizing the determination of protein in the Bradford assay (Bio-Rad Laboratories), calcium content was normalized according to the concentration of the protein.

- Alkaline phosphatase activity assay:
  Human aortic smooth muscle cells were treated for one week with phosphate supplementation as indicated. Agents with fresh media were added every two to three days. Human aortic smooth muscle cells were investigated to determine the alkaline phosphatase activity by utilizing the alkaline phosphatase activity colorimetric assay kit (Abcam, Cambridge, Massachusetts, USA) following the manufacturer's instructions. Normalizing the activity of alkaline phosphatase to total protein concentration was performed by the Bradford assay (Bio-Rad Laboratories).

- Quantitative RT-PCR:
  For the experiments with magnesium chloride, from human aortic smooth muscle cells and the aortic tissues of the C57BL/6 mice, total RNA was separated by using Trifast Reagent (Peqlab, Erlangen, Germany), adhering to the manufacturer's protocol. By using SuperScript III Reverse Transcriptase (Invitrogen) and oligo(dT)12–18 primers (Invitrogen, Carlsbad, California, USA), the reverse transcription of two milligram total RNA was achieved.

  The following mouse primers were used (5'-3' orientation):
  TN alkaline phosphatase (Alpl) fw: TGTGCCAGAGAAAGAGAGAGA;
  TN alkaline phosphatase (Alpl) rev: GTTTCAGGGCATTTTTCAAGGT;
  Casr fw: ATTCGACAC CTGTAACACCGT;
  Casr rev: GGAGCAGTTGCAGAACTCATC;
  Cbfa1 fw: AGAGTCAGATTACAGATCCCAGG;
  Cbfa1 rev: AGGAGGGGTAAGACTGGTCATA;

Col1a1 fw: ACCCGAGGTATGCTTGATCTG;
Col1a1 rev: CATTGCACG TCATCGCACAC;
Col3a1 fw: CCATTTGGAGAATGTTGTGCAAT;
Col3a1 rev: GGACATGATTCACAGATTCCAGG;
Fbn fw: GTGACACTTATGAGCGCCCTA;
Fbn rev: CCACTTGTC GCCAATCTTGTA;
Gapdh fw: AGGTCGGTGTGAACGGATTTG;
Gapdh rev: TGTAGACCATGTAGTTGAGGTCA;
Msx2 fw: TTCACCACATCCCAGCTTCTA;
Msx2 rev: TTGC AGTCTTTTCGCCTTAGC.
Also, the following human primers were used (5'-3' orientation):
TN alkaline phosphatase (ALPL) fw: GGACTGGTACTCAGACAACG;
TN alkaline phosphatase (ALPL) rev: GTAGGCGATGTCCTTACAGCC;
CASR fw: CCCTCTACGATTGCTGTGGTG;
CASR rev: AGGAGGCATAACTGACCTGGG;
CBFA1 fw: GCCTTCCACTCTCAGTAAGAAGA;
CBFA1 rev: GCCTGGGGTCTGAAAAAGGG;
GAPDH fw: GAGTCAACGGATTTGGTCGT;
GAPDH rev: GACAAGCTTCCCG TTCTCAG;
MSX2 fw: TGCAGAGCGTGCAGAGTTC;
MSX2 rev: GGCAGCATAGGTTTTGCAGC.
For the experiments investigating the role of GSK-3 in calcification, RNA was again separated from the tissues of the aorta by utilizing Trizol Reagent (Thermo Fisher Scientific) following the manufacturer's protocol. Reverse transcription of 2 µg RNA was conducted by utilizing SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) and oligo(dT)$_{12\text{-}18}$ primers (Thermo Fisher Scientific).
The following mouse primers were used (Thermo Fisher Scientific; 5'-3' orientation):
Alpl fw: TTGTGCCAGAGAAAGAGAGAGA;
Alpl rev: GTTTCAGGGCATTTTTCAAGGT;
Cbfa1 fw: AGAGTCAGATTACAGATCCCAGG;
Cbfa1 rev: AGGAGGGGTAAGACTGGTCATA;

Gapdh fw: AGGTCGGTGTGAACGGATTTG;
Gapdh rev: TGTAGACCATGTAGTTGAGGTCA;
Msx2 fw: TTCACCACATCCCAGCTTCTA;
Msx2 rev: TTGCAGTCTTTTCGCCTTAGC;
Pai1 fw: TTCAGCCCTTGCTTGCCTC;
Pai1 rev: ACACTTTTACTCCGAAGTCGGT;
Pit1 fw: TTTGACAAACTTCCTCTGTGGG;
Pit1 rev: GGACTTTCAGACGGACTAGACTT.

Quantitative RT-PCR was conducted with the iQ™ Sybr Green Supermix (Bio-Rad Laboratories) and iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories) in accordance with the manufacturer’s protocol.

The thermal cycling protocol used for RT-PCR was: initial denaturation for 2 minutes at 95°C followed by 39 cycles of denaturation for 15 seconds at 95°C and annealing/elongation for 45 seconds at 55°C. The specificity of the PCR products was confirmed by analysis of the melting curves run at the end of each RT-PCR experiment and measured for 2 seconds/step at 55-95°C (0.5°C increment). Every polymerase chain reaction was conducted in duplicate, and the relative mRNA fold alterations were assessed by the 2-ΔΔCt method utilizing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal reference and the relative mRNA fold changes were calculated.

- Western blot analysis:

For the magnesium experiments, right after washing human aortic smooth muscle cells by using phosphate buffered saline (PBS), cells were lysed by using ice-cold IP lysis buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with added phosphatase and protease inhibitor mixture (Thermo Fisher Scientific). Following 10 000 round-per-minute centrifugation for five minutes, thirty milligrams of protein were boiled in Roti-Load1 Buffer (Carl Roth GmbH, Karlsruhe, Germany) at 100 Celsius grades for ten minutes. By using SDS-polyacrylamide gels, the boiled proteins were separated and then moved to polyvinylidene difluoride membranes. The membranes were incubated overnight at 4 Celsius grades with the following primary antibodies: rabbit monoclonal anti-GAPDH antibody (diluted 1 : 1000, Cell Signaling, Danvers, Massachusetts, USA) or mouse monoclonal anti-CASR (diluted 1 : 500, Abcam) and then with secondary anti-mouse

horseradish peroxidase (HRP)-conjugated antibody and anti-rabbit HRP-conjugated antibody, respectively (diluted 1 : 1000, Cell Signaling) for one hour at normal room temperature. The binding of antibody was assessed with ECL detection reagent (Thermo Fisher Scientific). Quantifying the bands was conducted by utilizing ImageJ software (National Institutes of Health, Bethesda, MD, USA).

For western blot analysis of GSK-3 mice, the tissues of the aorta were lysed in ice-cold IP buffer (Thermo Fisher Scientific) with addition of complete phosphatase and protease inhibitor mixture (Thermo Fisher Scientific). After five minutes of centrifugation at ten thousand rounds per minute, the proteins were boiled in Roti-Load1 Buffer (Carl Roth GmbH) at hundred Celsius degrees for ten minutes. Same quantities of proteins were isolated on SDS-polyacrylamide gels and transferred to PVDF membranes.
The membranes were treated over the night at four Celsius degrees with primary antibodies: rabbit anti-phospho-GSK-3α/β (Ser21/9), rabbit anti-β-catenin, rabbit anti-GSK-3β, rabbit anti-GSK-3α or rabbit anti-Gapdh antibody (all diluted 1:1000, Cell Signaling) and right after that the incubation was conducted with secondary anti-rabbit HRP-conjugated antibody (diluted 1:1000, Cell Signaling) for one hour at room temperature.
In order to load the controls, in the stripping buffer (Thermo Fisher Scientific) the membranes were stripped at room temperature for ten minutes. Antibody binding was detected with ECL detection reagent (Thermo Fisher Scientific). Bands were evaluated by utilizing ImageJ software, and results are explained as the proportion of the whole amount of protein Gapdh and phosphorylated protein to total protein to Gapdh normalized to the control group.

- Statistics:

The data are depicted as arithmetic mean and SEM. The normality of the datasets were investigated by Shapiro-Wilk test. Datasets not meeting the assumption of normality according to Shapiro-Wilk test were log, sqrt or reciprocal transformed to provide normal distribution according to Shapiro-Wilk test. Statistical testing was conducted by a one-way ANOVA with Tukey or Games-Howell posthoc analysis, according to scedasticity. Non-Normal Data were analyzed by the Steel-Dwass test. Datasets of two groups were investigated by two-tailed T-Test. A p value of <0.05 was considered as statistically significant.

4. Results

4.1 Magnesium chloride experiments

4.1.1 $MgCl_2$ prevents phosphate-induced calcification and osteoinductive signaling in human aortic smooth muscle cells

In order to investigate the possible effects of magnesium chloride on vascular calcification, experiments were performed in phosphate-treated human aortic smooth muscle cells with or without adding magnesium chloride.

It was observed that the treatment with phosphate raised the deposition of calcium, but this deposition was significantly ameliorated with additional magnesium chloride treatment (Fig.1).

This finding indicates a preventive influence of magnesium chloride on vascular calcification.

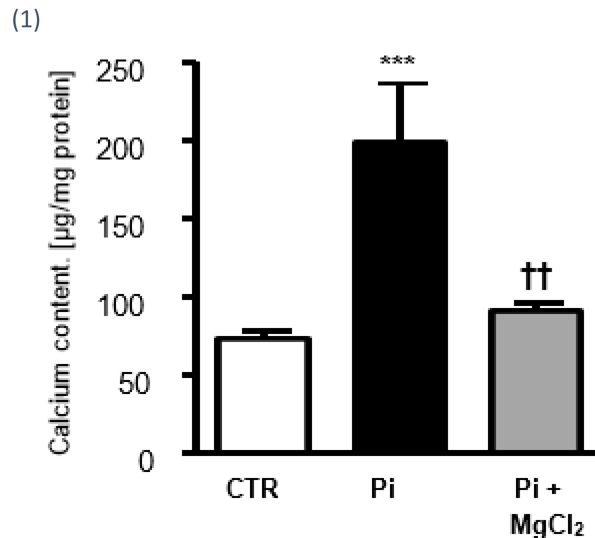

Fig.1. The average value (±) standard error of the mean of calcium content (n=6, µg/mg protein) in human aortic smooth muscle cells after treating for two weeks with CTR or with phosphate (Pi) with and without adding 1.5 mM/l magnesium chloride. ***($p<0.001$) statistically significant versus CTR treated human aortic smooth muscle cells; ††($p<0.01$) statistically significant versus human aortic smooth muscle cells treated only with phosphate.

Additional experiments investigated a possible association of reduced calcification during magnesium supplementation with the osteogenic reprogramming of vascular smooth muscle cells. To this end, the expression of markers of osteogenic transdifferentiation was investigated.

The mRNA expression of the osteogenic marker msh homeobox 2 (MSX2) was upregulated by 24 hours of 2 mM/l beta-glycerophosphate treatment, an upregulation that was markedly impaired by additional treatment with 1.5 mM/l magnesium chloride. (Fig.2).

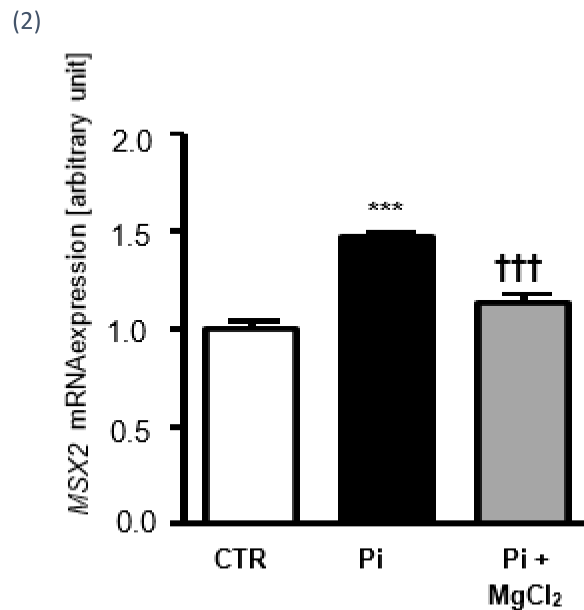

Fig.2. The average value (±) standard error of the mean (n=6; arbitrary units) of osteogenic marker (msh homeobox 2) mRNA expression in the human aortic smooth muscle cells after treatment with CTR conditions or phosphate (Pi) with and without adding magnesium chloride.

***($p<0.001$) statistically significant versus CTR treated human aortic smooth muscle cells; †††($p<0.001$) statistically significant versus human aortic smooth muscle cells treated only with phosphate.

In order to better characterize the osteogenic transdifferentiation, the expression levels of the osteogenic transcription factor core-binding factor a1 (CBFA1) were investigated. The phosphate treatment of primary human aortic smooth muscle cells induced elevated mRNA expression levels of CBFA1. The increased expression levels during elevated phosphate conditions were virtually abrogated by co-treatment of the vascular smooth muscle cells with magnesium chloride (Fig.3).

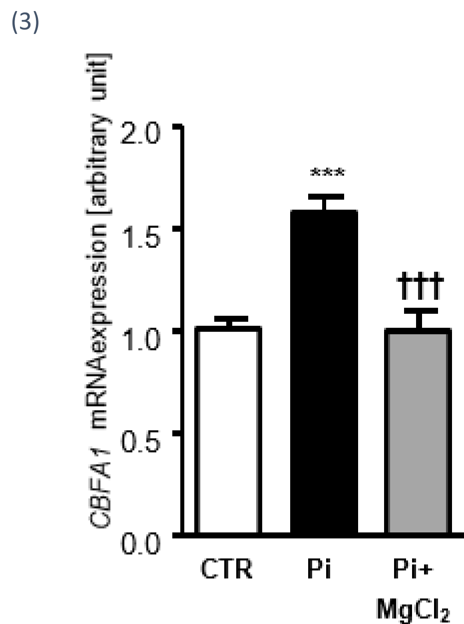

Fig.3. The average value (±) standard error of the mean (n=6; arbitrary units) of osteogenic marker (core-binding factor a 1) mRNA expression in the human aortic smooth muscle cells after treatment with CTR conditions or phosphate (Pi) with and without adding magnesium chloride.

***($p<0.001$) statistically significant versus CTR treated human aortic smooth muscle cells; †††($p<0.001$) statistically significant versus human aortic smooth muscle cells treated only with phosphate.

The next experiments investigated mRNA expression levels of tissue nonspecific alkaline phosphate (ALPL), a key mediator of vascular calcification.

In accordance with the previous observations, phosphate treatment was able to cause elevated mRNA expression levels of alkaline phosphatase in human aortic vascular smooth muscle cells, as compared to control conditions. The elevated expression of alkaline phosphatase was again normalized by additional treatment with magnesium chloride (Fig.4).

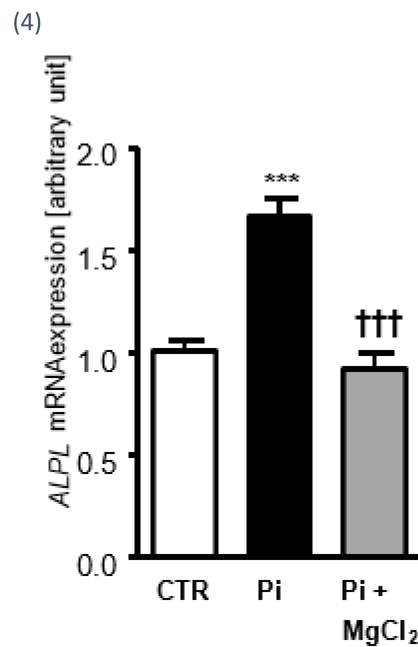

Fig.4. Average value (±) standard error of the mean (n=6; arbitrary units) of osteogenic marker (tissue-nonspecific alkaline phosphatase) mRNA expression in the human aortic smooth muscle cells after treatment with CTR conditions or phosphate (Pi) with and without adding magnesium chloride.

***($p<0.001$) statistically significant versus CTR treated human aortic smooth muscle cells; †††($p<0.001$) statistically significant versus human aortic smooth muscle cells treated only with phosphate.

To investigate the functional effects of the upregulated expression of alkaline phosphatase, additional measurements of alkaline phosphatase enzymatic activity was performed. Similar to the mRNA expression levels, phosphate treatment caused an increased activity of alkaline phosphatase in vascular smooth muscle cells as compared to control conditions. The phosphate-induced increase of alkaline phosphatase activity was again strongly blunted by co-treatment of magnesium chloride (Fig.5).

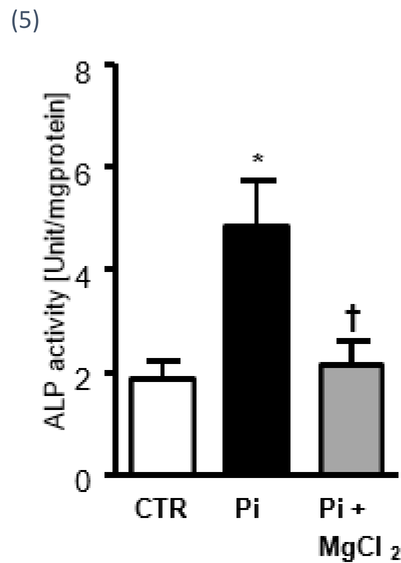

Fig.5. The average value (±) standard error of the mean of alkaline phosphatase activity (n=4, units/mg protein) in the human aortic smooth muscle cells after treatment with CTR conditions or phosphate (Pi) with and without adding magnesium chloride. *($p<0.05$) statistically significant versus CTR treated human aortic smooth muscle cells; †($p<0.05$) statistically significant versus human aortic smooth muscle cells treated only with phosphate.

4.1.2 $MgCl_2$ enhances calcium-sensing receptor expression in human aortic smooth muscle cells in a dose-dependent concept

Subsequent experiments investigated a possible underlying mechanism for the inhibitory effects of magnesium chloride on vascular calcification and osteogenic reprogramming. Therefore, the calcium-sensing receptor (CASR), on which magnesium could act as an agonist, was investigated as possible mediator. A first experiment aimed to determine mRNA expression of the CASR in response to magnesium chloride. As shown in Fig. 6, treatment of vascular smooth muscle cells with various concentrations of magnesium chloride dose-dependently upregulated the mRNA expression of CASR.

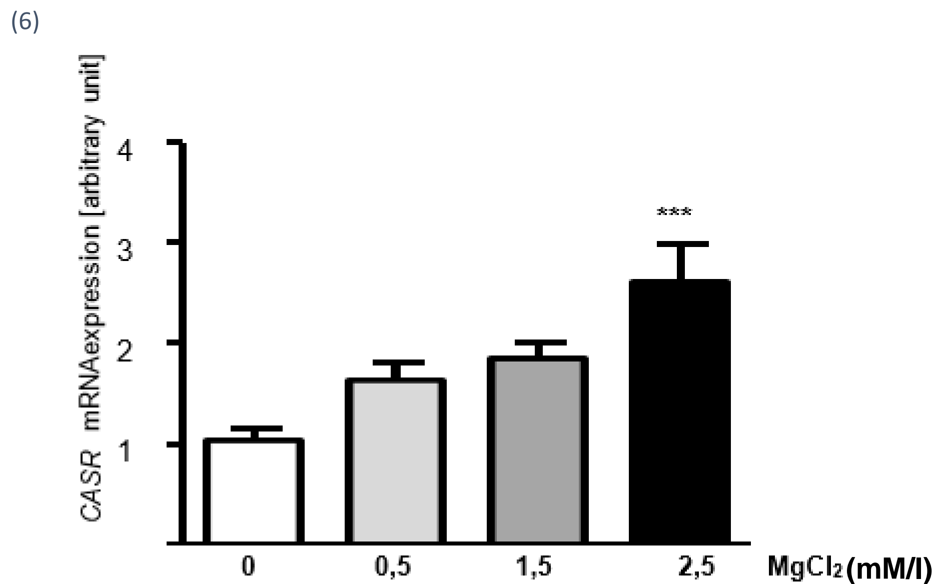

Fig.6. The average value (±) standard error of the mean (n=6; arbitrary units) of (calcium-sensing receptor) mRNA expression in human aortic smooth muscle cells after treatment for one day with CTR or with increasing concentrations of magnesium chloride (0.5 mM/l - 2.5 mM/l).

***($p<0.001$) statistically significant versus CTR treated human aortic smooth muscle cells.

4.1.3 Silencing of calcium-sensing receptor reduces the protective effects of $MgCl_2$ on phosphate-induced osteo-/chondrogenic transformation of human aortic smooth muscle cells

Further experiments addressed, whether the CASR may be a mediator of the protective effects of magnesium during high phosphate conditions. To this end, CASR expression was silenced by siRNA in vascular smooth muscle cells, with and without phosphate and magnesium treatment. Silencing of the CASR successfully reduced the mRNA expression of the CASR during all treatment conditions (Fig.7). Both phosphate and phosphate + magnesium treatment increased the CASR mRNA expression in negative control silenced cells. (calcium-sensing receptor) expression in human aortic smooth muscle cells.

(7)

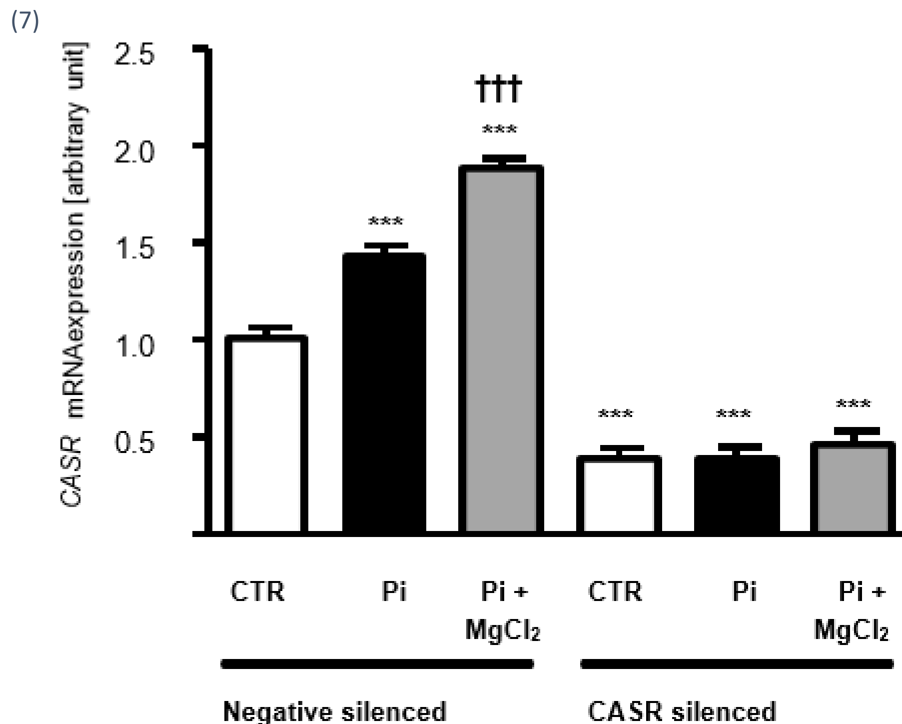

Fig.7. The average value (±) standard error of the mean (n=6; arbitrary units) of (calcium-sensing receptor) mRNA expression in human aortic smooth muscle cells after inhibiting for two days with negative CTR silenced RNA (Negative silenced) or (calcium-sensing receptor) silenced RNA (CASR silenced) and treating for one day with CTR or with 2 mM/l β-glycerophosphate (Pi) without or with treatment with 1.5 mM/l magnesium chloride. ***($p<0.001$) statistically significant versus Negative silenced human aortic smooth muscle cells; †††($p<0.001$) statistically significant between Negative silenced phosphate and phosphate +magnesium chloride treated human aortic smooth muscle cells.

Further experiments confirmed this observation on the protein expression level. As shown in (Fig.8), phosphate treatment alone did not significantly upregulate CASR-protein expression in vascular smooth muscle cells, but the combined treatment of phosphate plus magnesium induced a significant upregulation of CASR protein abundance in vascular smooth muscle cells.

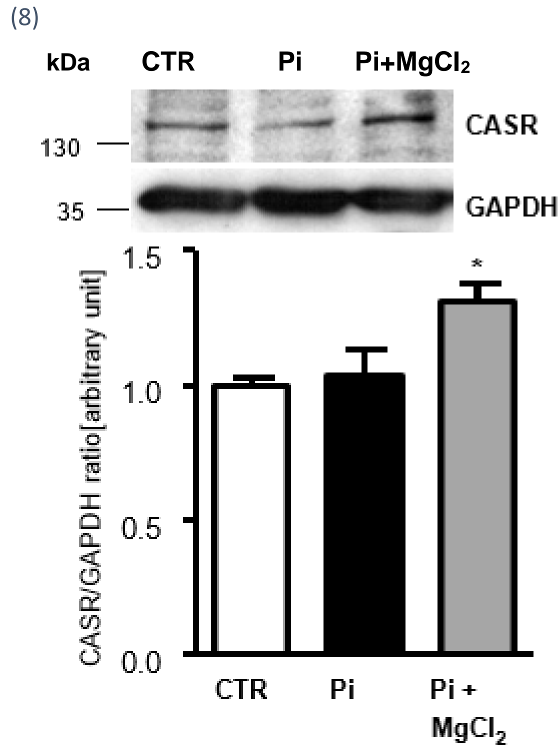

Fig.8. Representative original Western blots and the average value (±) standard error of the mean (n=4; arbitrary unit) of normalized CASR/GAPDH protein ratio in human aortic smooth muscle cells after treating for one day with CTR or with 2 mM/l β-glycerophosphate (Pi) without or with additional treatment with 1.5 mM/l magnesium chloride.

*($p<0.05$) statistically significant versus CTR treated human aortic smooth muscle cells.

In a series of further experiments, the effects of CASR-silencing during conditions of elevated phosphate and elevated phosphate with magnesium supplementation were investigated. As expected, long-term phosphate treatment caused calcification of vascular smooth muscle cells indicated by increased calcium deposition, irrespective of negative or CASR-silencing. Magnesium supplementation was able to blunt calcification in smooth muscle cells during negative control silencing, but was unable to prevent calcification during CASR-silencing (Fig.9).

(9)

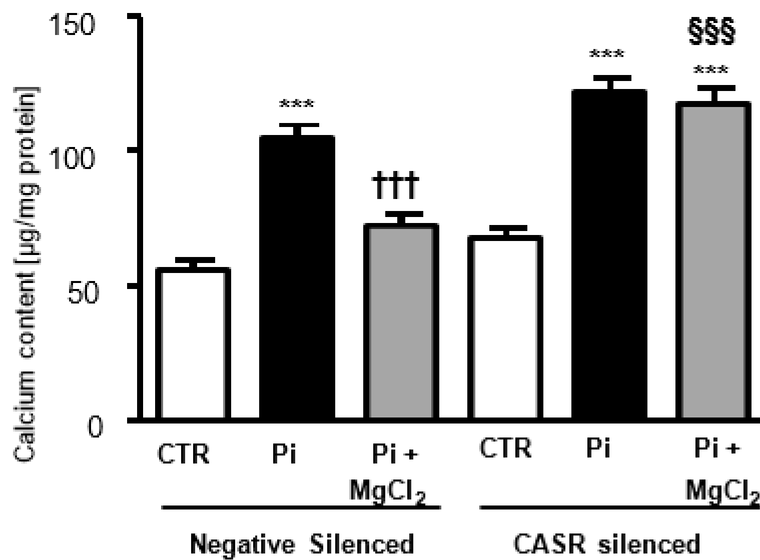

Fig.9. The average value (±) standard error of the mean of calcium content (n=6, µg/mg protein) in human smooth muscle cells after two weeks of negative CTR silencing or calcium-sensing receptor silencing, and treatment with CTR or with phosphate (Pi) without or with treatment with magnesium chloride.

***($p<0.001$) statistically significant versus Negative silenced human aortic smooth muscle cells; †††($p<0.001$) statistically significant between Negative silenced phosphate and phosphate + magnesium chloride treated human aortic smooth muscle cells; §§§($p<0.001$) statistically significant between negative silenced and CASR silenced phosphate + magnesium chloride treated human aortic smooth muscle cells.

Further experiments again investigated the mRNA expression of osteogenic markers under CASR conditions. In accordance with the previous observations, during negative silencing phosphate treatment elevated the mRNA expression levels of the osteogenic transcription factor MSX2 in vascular smooth muscle cells, an elevation blunted by cotreatment with magnesium chloride. However, during CASR-silencing, phosphate also induced an upregulation of MSX2, but magnesium chloride failed to significantly modulate the MSX2 mRNA expression levels. CASR-silencing itself was able to increase MSX2 mRNA expression levels in control treated cells (Fig.10).

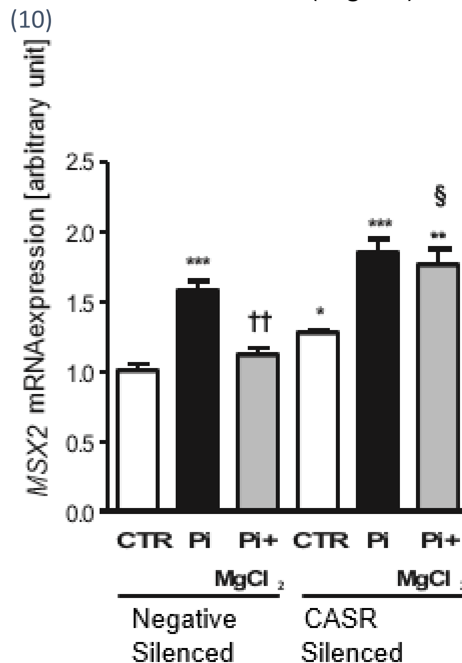

Fig.10. The average value (±) standard error of the mean (n=6; arbitrary unit) of osteogenic marker (msh homeobox 2) mRNA expression in human aortic smooth muscle cells following silencing for two days with negative CTR silenced RNA (Negative silenced) or calcium-sensing receptor silenced RNA and treating for one day with CTR or with 2 mM/l β-glycerophosphate (Pi) without or with treatment with 1.5 mM/l magnesium chloride. *($p<0.05$), **($p<0.01$), ***($p<0.001$) statistically significant versus Negative silenced human aortic smooth muscle cells; ††($p<0.01$) statistically significant between Negative silenced phosphate and phosphate + magnesium chloride treated human aortic smooth muscle cells; §($p<0.05$) statistically significant between negative silenced and CASR silenced phosphate + magnesium chloride treated human aortic smooth muscle cells.

Similar observations were made when measuring the mRNA expression levels of the osteogenic transcription factor CBFA1. The mRNA expression levels of the osteogenic transcription factor CBFA1 were elevated following phosphate treatment in negative silenced vascular smooth muscle cells, and this elevation was again blunted by cotreatment with magnesium chloride. In conditions of CASR-silencing, phosphate similarly induced an upregulation of CBFA1, but magnesium chloride was not able to significantly blunt the CBFA1 mRNA expression levels. (Fig.11).

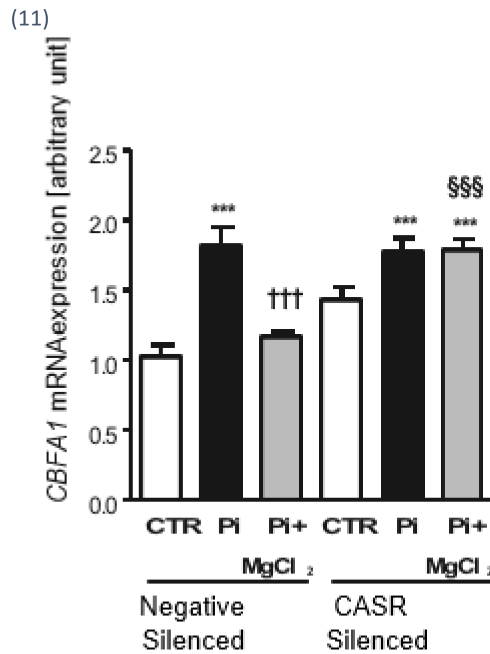

Fig.11. The average value (±) standard error of the mean (n=6; arbitrary unit) of osteogenic marker (core-binding factor a 1) mRNA expression in human aortic smooth muscle cells following silencing for two days with negative CTR silenced RNA (Negative silenced) or calcium-sensing receptor silenced RNA and treatment for one day with CTR or with 2 mM/l β-glycerophosphate (Pi) without or with treatment with 1.5 mM/l magnesium chloride.

***($p<0.001$) statistically significant versus Negative silenced human aortic smooth muscle cells; †††($p<0.001$) statistically significant between Negative silenced phosphate and phosphate +magnesium chloride treated human aortic smooth muscle cells; §§§($p<0.001$) statistically significant between negative silenced and CASR silenced phosphate +magnesium chloride treated human aortic smooth muscle cells.

A comparable effect was again observed when determining the expression levels of the tissue nonspecific alkaline phosphatase (ALPL). Increased phosphate concentrations markedly increased the tissue-nonspecific alkaline phosphatase mRNA expression in negative control-silenced human aortic smooth muscle cells, and this effect was notably inhibited by the presence of magnesium chloride.

This inhibitory effect of magnesium chloride on phosphate-induced ALPL mRNA expression was again abrogated during conditions of CASR-silencing (Fig.12).

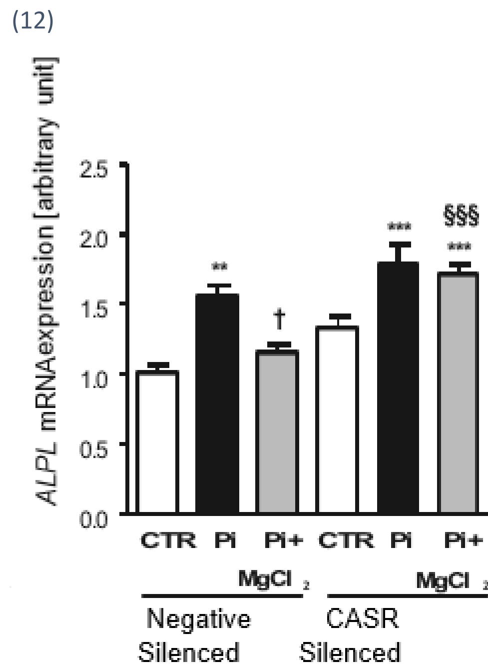

Fig.12. Average value (±) standard error of the mean (n=6; arbitrary unit) of osteogenic marker (tissue-nonspecific alkaline phosphatase) mRNA expression in human aortic smooth muscle cells following silencing for two days with negative CTR silenced RNA (Negative silenced) or calcium-sensing receptor silenced RNA and treating for one day with CTR or with 2 mM/l β-glycerophosphate (Pi) without or with treating with 1.5 mM/l magnesium chloride. **($p<0.01$), ***($p<0.001$) statistically significant versus Negative silenced human aortic smooth muscle cells; †($p<0.05$) statistically significant between Negative silenced phosphate and phosphate + magnesium chloride treated human aortic smooth muscle cells; §§§($p<0.001$) statistically significant between Negative silenced and CASR silenced phosphate + magnesium chloride treated human aortic smooth muscle cells.

4.1.4 Calcium-sensing receptor agonist mimics and antagonist reverse the effects of magnesium chloride on phosphate-induced osteo-/chondrogenic transformation of human aortic smooth muscle cells

To further confirm the critical role of the calcium-sensing receptor in the influence of magnesium chloride on osteo/chondrogenic signaling and vascular calcification, human aortic smooth muscle cells were treated with phosphate with adding or without adding magnesium chloride in both presence or absence of calcium-sensing receptor agonist gadolinium trichloride or of the calcium-sensing receptor inhibitor NPS-2143. The presence of magnesium chloride reduced the phosphate-induced calcium accumulation in human aortic smooth muscle cells, this effect was imitated by the calcium-sensing receptor agonist gadolinium trichloride and abrogated by additional treatment with the calcium-sensing receptor inhibitor NPS-2143 (Fig.13).

(13)

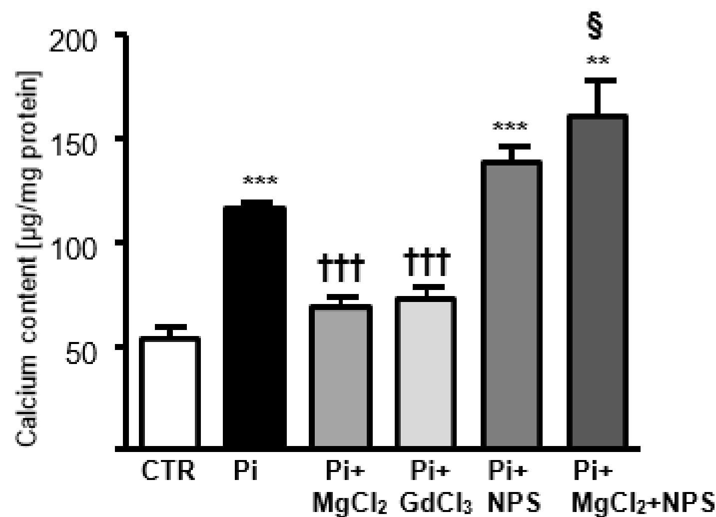

Fig.13. The average value (±) standard error of the mean of calcium content (n=6, µg/mg protein) in human aortic smooth muscle cells after treating for two weeks with CTR or with 3 mM/l sodium phosphate buffer (Pi) without or with adding 1.5 mM/l magnesium chloride, 50 µM GdCl3, 100 nM/l NPS-2143 (NPS) or 1.5 mM/l magnesium chloride with 100 nM/l NPS-2143. **($p<0.01$), ***($p<0.001$) statistically significant versus CTR treated human aortic smooth muscle cells; †††($p<0.001$) statistically significant versus human aortic smooth muscle cells treated with phosphate only; §($p<0.05$), statistically significant between human aortic smooth muscle cells treated with phosphate + magnesium chloride and phosphate + magnesium chloride + NPS.

Furthermore, the phosphate-induced, osteogenic marker MSX2 (msh homeobox 2) mRNA expression was notably decreased by treating with either magnesium chloride or gadolinium trichloride, indicating that gadolinium mimics the effects of magnesium on prevention of osteogenic transdifferentiation.

The inhibitory effect of magnesium chloride on phosphate-induced mRNA expression of MSX2 was again blocked by addition of the CASR antagonist NPS-2143 (Fig.14).

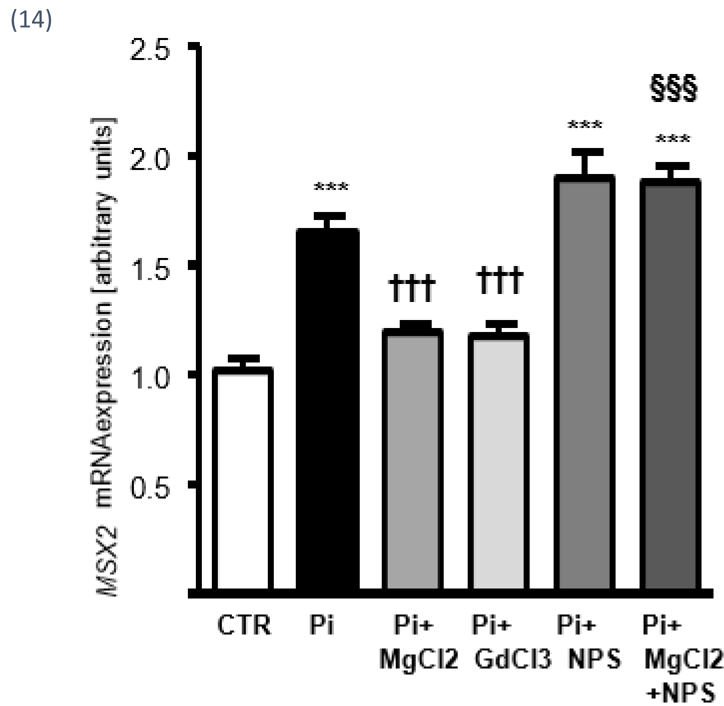

Fig.14. Average value (±) standard error of the mean (n=8; arbitrary units) of (msh homeobox 2) mRNA expression in human aortic smooth muscle cells after treating for one day with CTR or with 2 mM/l β-glycerophosphate (Pi) without or with adding 1.5 mM/l magnesium chloride, 50 µM GdCl3, 100 nM/l NPS-2143 (NPS) or 1.5 mM/l magnesium chloride with 100 nM/l NPS-2143.

***(p<0.001) statistically significant versus CTR treated human aortic smooth muscle cells; †††(p<0.001) statistically significant versus human aortic smooth muscle cells treated with phosphate only; §§§(p<0.001) statistically significant between human aortic smooth muscle cells treated with phosphate + magnesium chloride and phosphate + magnesium chloride + NPS.

Again, similar findings were also observed when investigating the mRNA expression of the osteogenic transcription factor CBFA1 (core-binding factor a 1). The phosphate-induced increase of mRNA expression of the osteogenic marker CBFA1 was remarkably decreased by treating with either magnesium chloride or gadolinium trichloride. The protective effects of magnesium chloride were again abrogated by the CASR-antagonist NPS (Fig.15).

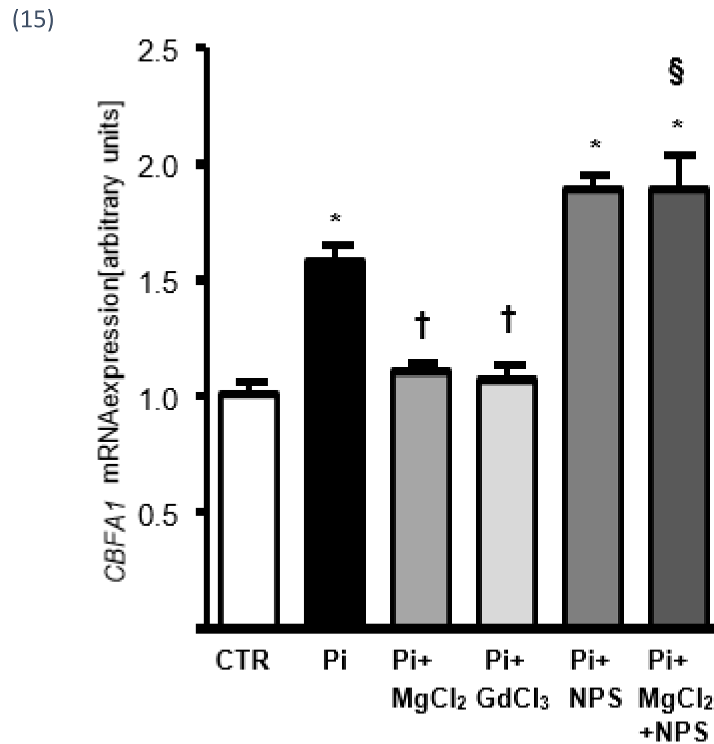

Fig.15. The average value (±) standard error of the mean (n=8; arbitrary units) of (core-binding factor a 1) mRNA expression in human aortic smooth muscle cells after treating for one day with CTR or with 2 mM/l β-glycerophosphate (Pi) without or with adding 1.5 mM/l $MgCl_2$, 50 μM GdCl3, 100 nM/l NPS-2143 (NPS) or 1.5 mM/l magnesium chloride with 100 nM/l NPS-2143.

*($p<0.05$) statistically significant versus CTR treated human aortic smooth muscle cells; †($p<0.05$), statistically significant versus human aortic smooth muscle cells treated with phosphate only; §($p<0.05$) statistically significant between human aortic smooth muscle cells treated with phosphate + magnesium chloride and phosphate + magnesium chloride + NPS.

Furthermore, in this experiment it was observed that gadolinium trichloride exerts comparable effects as magnesium chloride on the phosphate-induced mRNA expression of the tissue-nonspecific alkaline phosphatase, as supplementation of both in the cell culture medium could blunt the phosphate-induced ALPL mRNA expression.
Additional treatment with the calcium-sensing receptor inhibitor NPS-2143 abrogated the blunted mRNA expression induced by co-treatment with magnesium under high-phosphate conditions (Fig.16).

(16)

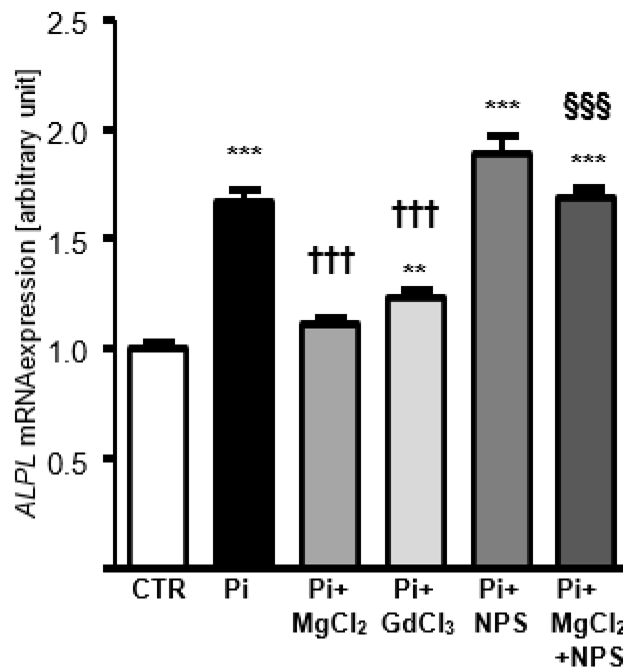

Fig.16. Average value (±) standard error of the mean (n=8; arbitrary units) of (tissue-nonspecific alkaline phosphatase) mRNA expression in human aortic smooth muscle cells after treatment for one day with CTR or with 2 mM/l β-glycerophosphate (Pi) without or with adding 1.5 mM/l magnesium chloride, 50 μM GdCl3, 100 nM/l NPS-2143 (NPS) or 1.5 mM/l magnesium chloride with 100 nM/l NPS-2143.

($p<0.01$), *($p<0.001$) statistically significant versus CTR treated human aortic smooth muscle cells; †††($p<0.001$) statistically significant versus human aortic smooth muscle cells treated with phosphate only; §§§($p<0.001$) statistically significant between human aortic smooth muscle cells treated with phosphate + magnesium chloride and phosphate + magnesium chloride + NPS.

4.1.5 $MgCl_2$ inhibits hydroxyapatite-induced osteoinductive signaling in human aortic smooth muscle cells

Besides the CASR-mediated effects, Magnesium may be effective by inhibiting directly the precipitation of calcium and phosphate. Therefore, additional experiments were conducted with treating human aortic smooth muscle cells with preformed hydroxyapatite. Hydroxyapatite, similar to phosphate, upregulated the mRNA expression of the CASR in cultured smooth muscle cells. Addition of magnesium further augmented the increase of CASR mRNA expression (Fig.17).

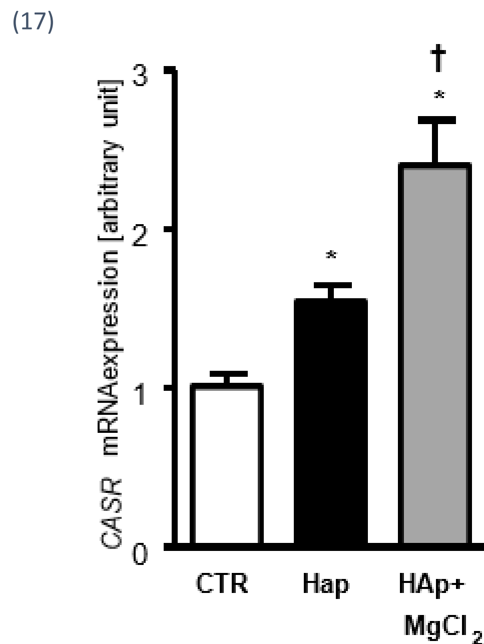

Fig.17. The average value (±) standard error of the mean (n=6; arbitrary units) of calcium-sensing receptor mRNA expression in human aortic smooth muscle cells after treating for one day with CTR or with 5 µg/ml hydroxyapatite without or with adding 1.5 mM/l magnesium chloride.

*($p<0.05$) statistically significant versus CTR treated human aortic smooth muscle cells; †($p<0.05$) statistically significant versus human aortic smooth muscle cells treated with only hydroxyapatite.

Further experiments addressed the effects of magnesium during hydroxyapatite treatment on mRNA expression of osteogenic marker expression. Similar to phosphate treatment, hydroxyapatite treatment increased the osteogenic marker msh homeobox 2 mRNA expression in human aortic smooth muscle cells. These effects were significantly reduced by additional treatment with magnesium chloride (Fig.18).

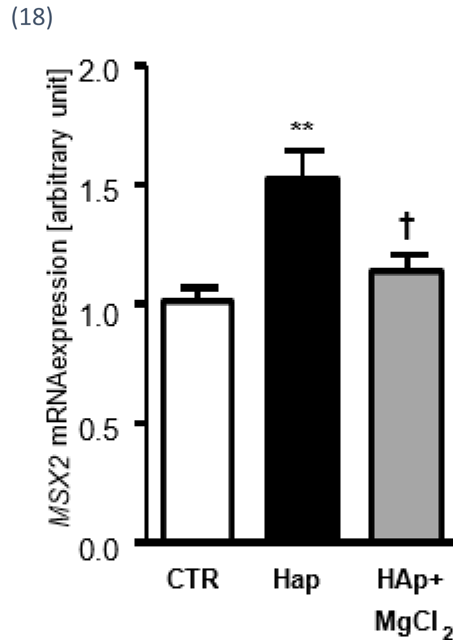

Fig.18. The average value (±) standard error of the mean (n=6; arbitrary units) of msh homeobox 2 mRNA expression in human aortic smooth muscle cells after treating for one day with CTR or with 5 µg/ml hydroxyapatite without or with adding 1.5 mM/l magnesium chloride.

**($p<0.01$) statistically significant versus CTR treated human aortic smooth muscle cells; †($p<0.05$) statistically significant versus human aortic smooth muscle cells treated with only hydroxyapatite.

Moreover, hydroxyapatite treatment strongly upregulated the osteogenic marker core-binding factor a 1 mRNA expression in human aortic smooth muscle cells. These effects were again significantly blunted by the presence of magnesium chloride in the cell culture medium (Fig.19).

(19)

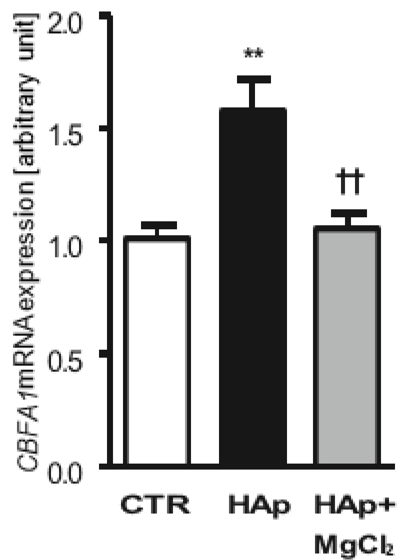

Fig.19. The average value (±) standard error of the mean (n=6; arbitrary units) of core-binding factor a 1 mRNA expression in human aortic smooth muscle cells after treating for one day with CTR or with 5 μg/ml hydroxyapatite without or with adding 1.5 mM/l magnesium chloride.

**($p<0.01$) statistically significant versus CTR treated human aortic smooth muscle cells; ††($p<0.01$) statistically significant versus human aortic smooth muscle cells treated with only hydroxyapatite.

Furthermore, the hydroxyapatite treatment also increased the tissue-nonspecific alkaline phosphatase mRNA expression in human aortic smooth muscle cells. Similar to the expression of the other osteogenic markers, the osteogenic effects of hydroxyapatite were blunted by additional treatment with magnesium chloride (Fig.20).

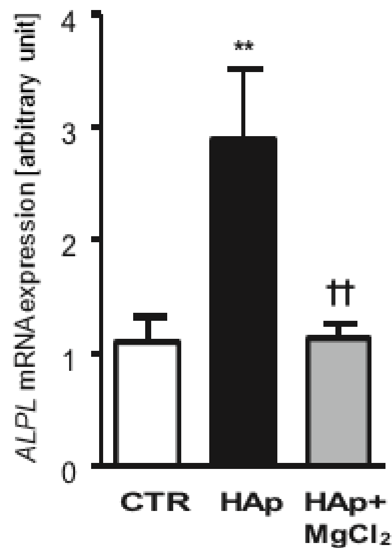

Fig.20. Average value (±) standard error of the mean (n=6; arbitrary units) of (tissue-nonspecific alkaline phosphatase) mRNA expression in human aortic smooth muscle cells after treatment for one day with CTR or with 5 µg/ml hydroxyapatite without or with adding 1.5 mM/l magnesium chloride.

**($p<0.01$) statistically significant versus CTR treated human aortic smooth muscle cells; ††($p<0.01$) statistically significant versus human aortic smooth muscle cells treated with only hydroxyapatite.

This observation was also replicated by the measurement of the alkaline phosphatase activity. Hydroxyapatite treatment could increase the alkaline phosphatase activity in human aortic smooth muscle cells and the effects were significantly reduced by additional treatment with magnesium chloride (Fig.21).

(21)

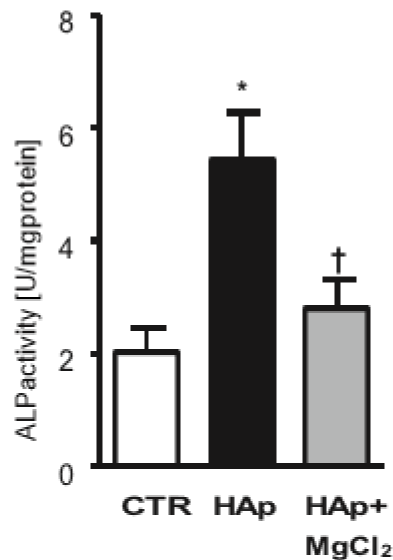

Fig.21. Average value (±) standard error of the mean of alkaline phosphatase activity (n=4, units/mg protein) in human aortic smooth muscle cells after treatment for one week with CTR or with 5 µg/ml hydroxyapatite without or with additional adding 1.5 mM/l magnesium chloride.

*($p<0.05$) statistically significant versus CTR treated human aortic smooth muscle cells; †($p<0.05$) statistically significant versus human aortic smooth muscle cells treated with only hydroxyapatite.

4.1.6 Magnesium chloride ameliorates vascular calcification and osteoinductive signaling during vitamin D overload-induced calcification

To confirm an inhibitory effect of magnesium on vascular calcification in the intact organism, experiments were performed in the vitamin D (cholecalciferol) overload model of vascular calcification in mice with and without magnesium feeding in the drinking water. The induction of calcification was associated with elevated calcium and fibroblast growth factor 23 (FGF23) levels in the serum of the mice. Phosphate levels were not significantly modified by vitamin D overload in the mice. Magnesium chloride feeding had no significant effects on any of the parameters (Table 1).

	CTR	vD	vD + $MgCl_2$	
Calcium (mg/dl)	8.6 +/- 0.2	21.4 +/- 1.7***	19.1 +/- 1.3***	n=7–9
Phosphate (mg/dl)	7.6 +/- 0.3	7.6 +/- 0.9	7.4 +/- 0.7	n=7–9
FGF23 C-term (pg/ml)	465 +/- 32	84330 +/- 12380***	62827 +/- 5282***	n=6–9

Table 1: Plasma calcium, phosphate and FGF23 levels during Vitamin D overload
Arithmetic means and SEM of plasma calcium, phosphate and FGF23 levels in mice receiving vehicle (CTR), vitamin D overload (vD) or vitamin D overload with Magnesium chloride feeding (vD + $MgCl_2$).
***P<0.001 statistically significant vs. control mice.

As a next step, the effects of magnesium feeding on vascular calcification induced by vitamin D (cholecalciferol) overload was investigated.

As shown in (Fig.22), cholecalciferol treatment induced calcium accumulation in the wall of the aortic arch as a measure of calcification when compared to control treated mice. The calcification was significantly blunted in the mice receiving magnesium containing drinking water.

(22)

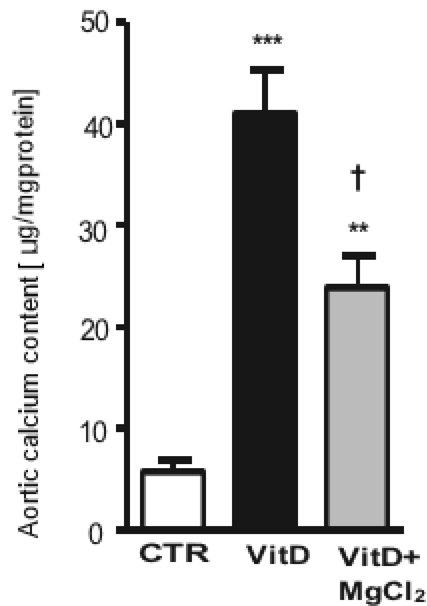

Fig.22. Average value (±) standard error of the mean of calcium content (n=5-7, µg/mg protein) in the aortic arch of mice receiving vehicle CTR or high-dosed cholecalciferol vitD without or with adding magnesium chloride.

($p<0.01$), *($p<0.001$) statistically significant versus CTR mice; †($p<0.05$), statistically significant versus vitD treated mice.

The high dosed cholecalciferol injection increased the calcium-sensing receptor mRNA expression in aortic tissues of the mice. This upregulation was significantly enhanced in mice receiving magnesium containing drinking water. Thus, the mRNA expression levels of the CASR were significantly higher in mice receiving vitamin D plus magnesium chloride than those receiving vitamin D alone (Fig.23).

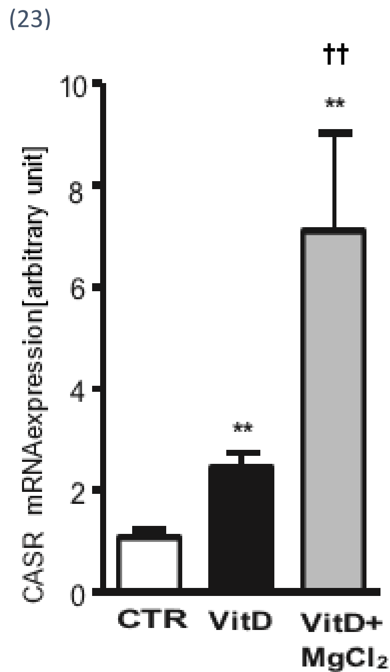

Fig.23. Average value (±) standard error of the mean of (calcium-sensing receptor) (n=7-9; arbitrary units) mRNA expression in aortic tissue of mice receiving vehicle CTR or high-dosed cholecalciferol vitD with or without adding magnesium chloride.
**($p<0.01$) statistically significant versus CTR mice; ††($p<0.01$) statistically significant versus vitD treated mice.

In addition, the high-dosed vitamin D treatment increased the aortic mRNA expression of the osteogenic marker msh homeobox 2 (MSX2) in the mice.

Feeding of magnesium chloride in the drinking water was able to significantly blunt the vitamin D-induced upregulation of the mRNA expression in the aortic tissue of mice (Fig.24).

(24)

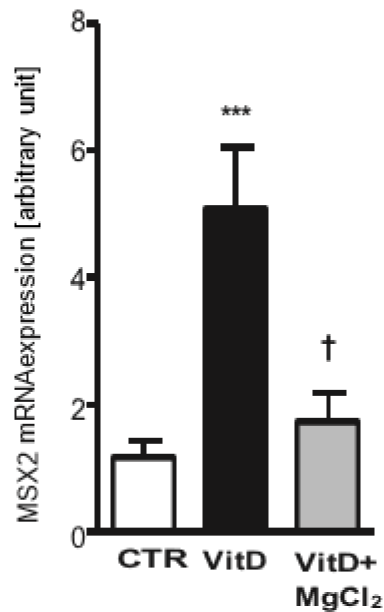

Fig.24. The average value (±) standard error of the mean of (msh homeobox 2) (n=7-9; arbitrary units) mRNA expression in aortic tissue of mice receiving vehicle CTR or high-dosed cholecalciferol vitD with or without adding magnesium chloride.
***($p<0.001$) statistically significant versus CTR mice; †($p<0.05$) statistically significant versus vitD treated mice.

The osteogenic effects of high dosed vitamin D treatment were again confirmed by the aortic mRNA expression of the osteogenic marker core-binding factor a1 (CBFA1). Vitamin D overload caused an increased mRNA expression of CBFA1 in the aortic tissue of mice. Magnesium supplementation in the drinking water was again able to significantly blunt the elevated CBFA1 expression levels in the mouse aortic tissue following Vitamin D overload (Fig.25).

(25)

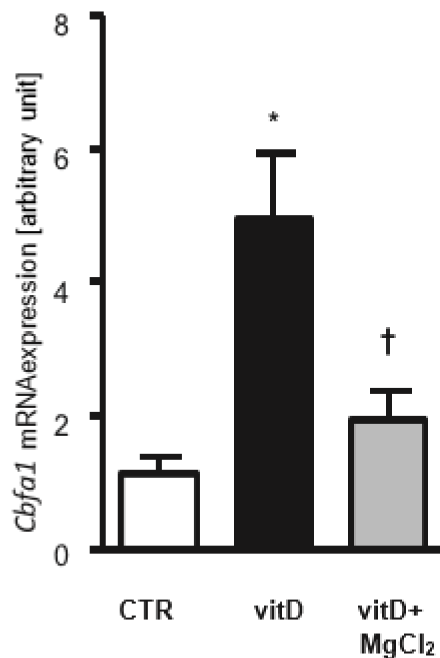

Fig.25. Average value (±) standard error of the mean of (core-binding factor a 1) (n=7-9; arbitrary units) mRNA expression in aortic tissue of mice receiving vehicle CTR or high-dosed cholecalciferol vitD with or without adding magnesium chloride.
*($p<0.05$) statistically significant versus CTR mice; †($p<0.05$) statistically significant versus vitD treated mice.

The osteogenic transdifferentiation of aortic tissue was further shown by the mRNA expression levels of ALPL. The treatment with high-dosed vitamin D increased the aortic mRNA expression of the osteogenic marker tissue-nonspecific alkaline phosphatase ALPL. Once again, the increased expression of ALPL in mouse aortic tissue induced by cholecalciferol overload was significantly blunted in mice receiving magnesium chloride in the drinking water (Fig.26).

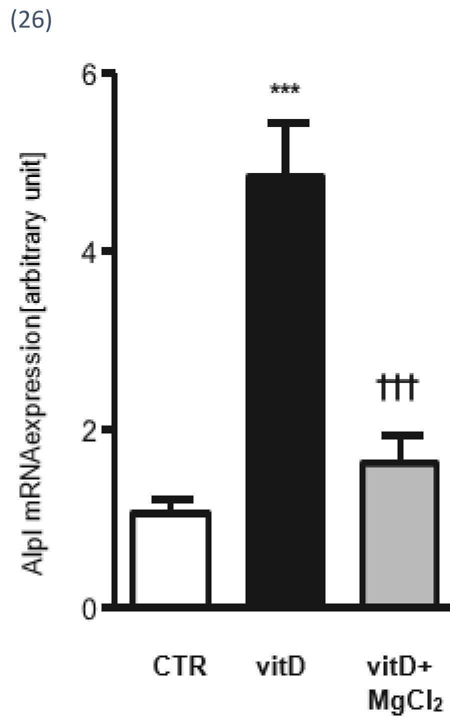

Fig.26. Average value (±) standard error of the mean of (tissue-nonspecific alkaline phosphatase) (n=7-9; arbitrary units) mRNA expression in aortic tissue of mice receiving vehicle CTR or high-dosed cholecalciferol vitD with or without adding magnesium chloride.

***($p<0.001$) statistically significant versus CTR mice; †††($p<0.001$) statistically significant versus vitD treated mice.

4.1.7 Magnesium chloride ameliorates expression of vascular stiffness markers during vitamin D3 overload-induced calcification.

Additional experiments addressed, whether the impaired osteogenic transdifferentiation in mouse aortic tissue following vitamin D overload induced by magnesium feeding was also associated with a modulated expression of markers of vascular stiffness.
High-dosed vitamin D treatment significantly elevated the aortic mRNA expression of the vascular stiffness marker collagen type I in mice. The feeding with magnesium was able to significantly ameliorate the elevated collagen type I mRNA expression levels (Fig.27).

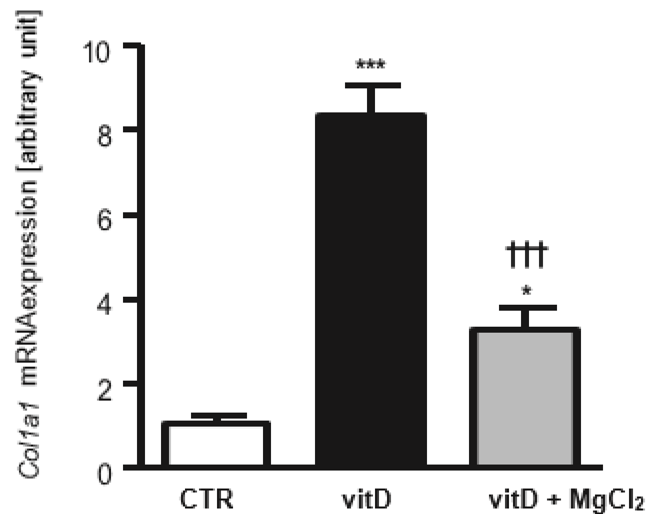

Fig.27. The average value (±) standard error of the mean of Col1a1 (n=7-9; arbitrary units) mRNA expression in aortic tissue of mice receiving CTR or high-dosed cholecalciferol vitD without or with adding magnesium chloride.
*(p<0.05), ***(p<0.001) statistically significant versus CTR mice; †††(p<0.001) statistically significant versus vitD injected mice.

Similar observations were made on the mRNA expression levels of collagen type III. High-dosed vitamin D treatment further elevated the aortic mRNA expression of the stiffness marker collagen type III.

Adding magnesium chloride to the drinking water was again able to reduce the elevated aortic mRNA expression levels of the stiffness marker collagen type III (Fig.28).

(28)

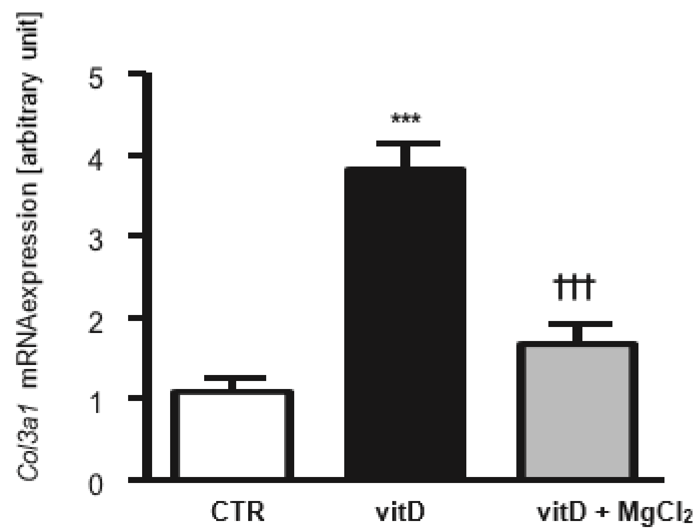

Fig.28. Average value (±) standard error of the mean of Col3a1 (n=7-9; arbitrary units) mRNA expression in aortic tissue of mice receiving CTR or high-dosed cholecalciferol vitD without or with adding magnesium chloride.

***($p<0.001$) statistically significant versus CTR mice; †††($p<0.001$) statistically significant versus vitD injected mice.

To confirm the effects of magnesium chloride on expression of extracellular matrix components and stiffness markers during vitamin D overload, the expression of fibronectin was determined.

High-dosed vitamin D treatment significantly elevated the aortic mRNA expression of the stiffness marker fibronectin. The elevated expression levels of fibronectin were again significantly blunted by magnesium chloride supplementation in the drinking water of the mice (Fig.29).

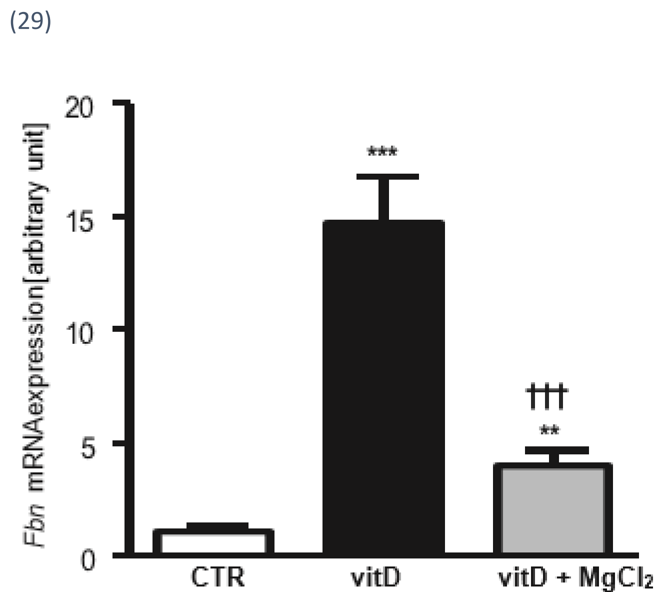

Fig.29. The average value (±) standard error of the mean of fibronectin (n=7-9; arbitrary units) mRNA expression in aortic tissue of mice receiving (CTR) or high-dosed cholecalciferol (vitD) without or with adding magnesium chloride.

(p<0.01), *(p<0.001) statistically significant versus CTR mice; †††(p<0.001) statistically significant versus vitD injected mice.

4.2 GSK3 activation/inhibition experiments

4.2.1 Role of AKT/SGK-dependent phosphorylation of GSK-3α/β in vascular calcification and aortic osteo-inductive signaling

To better understand the cellular signaling pathways of vascular calcification, another set of experiments addressed the role of the glycogen synthetase kinase 3 during vascular calcification. More specifically, it was investigated, whether phosphorylation of GSK-3α/β by the kinases SGK or AKT may be involved in osteogenic transdifferentiation of vascular tissue. To this end, the osteogenic reprogramming and vascular calcification was investigated in mice lacking the SGK/AKT dependent phosphorylation sites on GSK-3α/β (GSK-3α/β knock-in mice (ki), carrying a Ser-Ala GSK-3α/β21A/21A/9A/9A mutation, thus expressing AKT/SGK-resistant GSK3α/β) and corresponding control mice (wt). These mice were subjected again to vitamin D overload, in order to analyse the calcifying tissues. The induction of calcification was again associated with elevated calcium levels in the serum of the mice. No difference between the genotypes was noted. Phosphate levels were reduced by vitamin D overload in the mice an effect reaching statistical significance only in the GSK3 ki mice (Table 2).

	gsk-3 WT	gsk-3 KI	gsk-3 WT vD	gsk-3 KI vD	
Calcium (mg/dl)	9.2 +/- 0.6	9.4 +/- 0.5	21.8 +/- 0.9 ***	21.8 +/- 0.9 ***	n=6–9
Phosphorus (mg/dl)	8.2 +/- 0.3	7.6 ± 0.7	6.5 +/- 0.4	6.1 +/- 0.3 *	n=6–9

Table 2: arithmetic means and SEM of serum calcium and phosphorus concentrations in the gsk-3KI mice and corresponding gsk-3WT mice after vehicle treatment or vitamin D overload (vD). *(p<0.05), ***(p<0.001) statistically significant vs. control gsk-3WT mice.

As a next step, the calcification of vascular tissue was analyzed in the aortic arch of the mice. As depicted in figure 30, vitamin D overload vascular calcification in the mice, as evidenced by increased calcium content of the aortic arch, compared to mice receiving vehicle only. However, the calcium content was significantly lower in gsk3 ki mice as compared to gsk3 wt mice following vitamin D overload. (Fig.30).

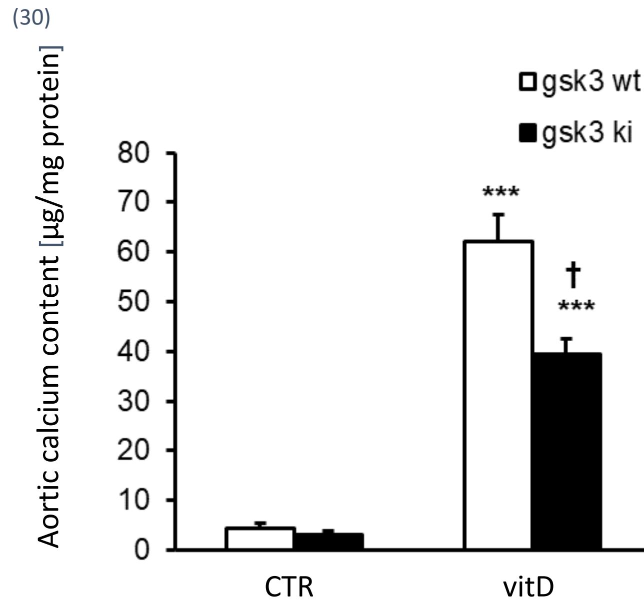

Fig.30. The average value (±) standard error of the mean of calcium content (n=6–9; µg/mg protein) in the aortic arch of gsk-3 knock-in mice (ki) and corresponding gsk-3 wild type mice (wt) receiving CTR or high dosed vitD.

***($p<0.001$) statistically significant versus CTR gsk-3 wild type mice; †($p<0.05$), statistically significant versus vitD injected gsk-3 wild type mice.

In a next series of experiments, the osteogenic transdifferentiation was investigated, as determined by mRNA expression of osteogenic markers, quantified by RT-PCR. As a first measurement, the expression levels of the osteogenic transcription factor msh homeobox 2 (MSX2) was investigated.

Consistent with previous observations, vitamin D overload increased expression of MSX2 in vitamin D treated mice. Accordingly, MSX2 mRNA expression levels were higher in vitamin D overload treated mice as compared to control mice. However, the upregulation of MSX2 mRNA by vitamin D overload was significantly blunted in the GSK3 ki mice as compared to GSK3 wt mice (Fig.31).

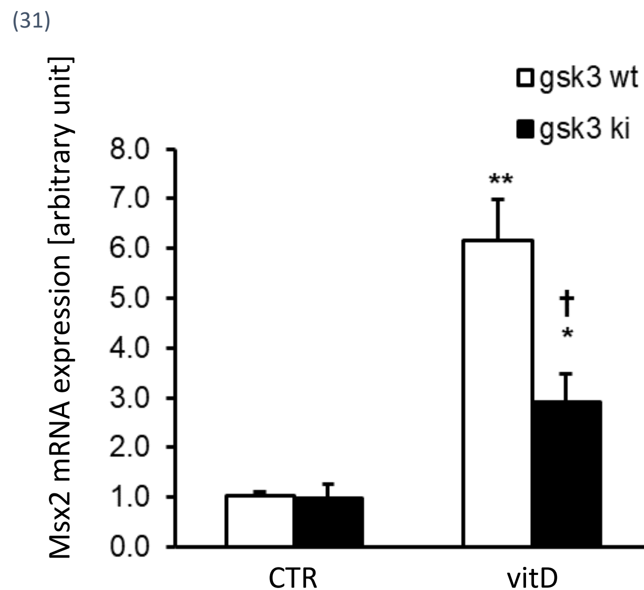

Fig.31. The average value (±) standard error of the mean of (n=6–9; arbitrary units) of osteogenic marker (msh homeobox 2) relative mRNA expression in aortic tissue of gsk-3 knock-in mice (ki) and corresponding gsk-3 wild type mice (wt) receiving CTR or high dosed (vitD).

*($p<0.05$), **($p<0.01$) statistically significant versus CTR gsk-3 wild type mice; †($p<0.05$), statistically significant versus vitD injected gsk-3 wild type mice.

As a next step, the expression of the osteogenic transcription factor core-binding factor a1 (Cbfa1) in aortic tissues was measured. The cholecalciferol overload was again associated with an increased mRNA expression of Cbfa1 in GSK3 wt mice, when compared to control treated wildtype mice. In accordance with the observations on MSX2 mRNA expression levels, the upregulation of Cbfa1 mRNA in aortic tissue following vitamin D overload was strongly blunted in GSK3 ki mice. Thus, the expression levels of Cbfa1 mRNA were significantly lower in GSK3 ki as compared to GSK3 wt mice following vitamin D overload (Fig 32).

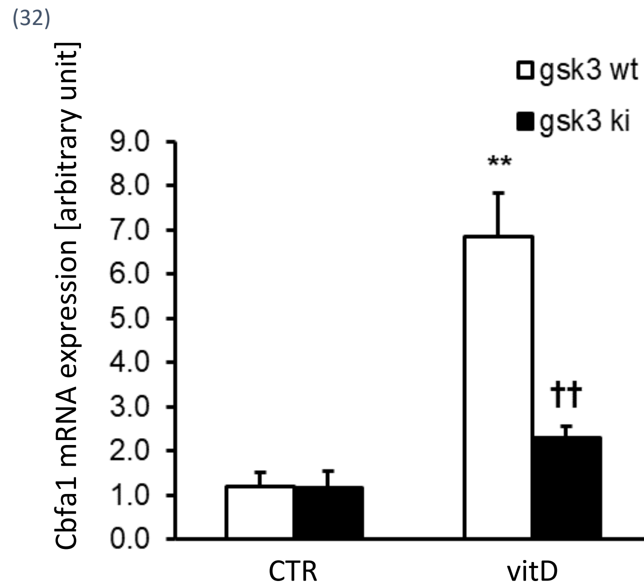

Fig.32. The average value (±) standard error of the mean of (n=6–9; arbitrary units) of osteogenic marker (core-binding factor a 1) relative mRNA expression in aortic tissue of gsk-3 knock-in mice (ki) and corresponding gsk-3 wild type mice (wt) receiving CTR or high dosed vitD.

**(p<0.01) statistically significant versus CTR gsk-3 wild type mice; ††(p<0.01) statistically significant versus vitD injected gsk-3 wild type mice.

To further characterize the osteogenic response of vascular tissue in GSK3 ki mice, the osteogenic enzyme tissue-nonspecific alkaline phosphatase was measured as an indicator of vascular osteo-/chondrogenic transdifferentiation. The mRNA expression levels of the tissue-nonspecific alkaline phosphatase (Alpl) were significantly increased in both genotypes following vitamin D overload. But again, this increase of Alpl mRNA expression was blunted in GSK3 ki mice, and the mRNA expression levels following vitamin D overload were significantly lower in GSK3 ki mice as compared to GSK3 wt mice (Fig.33).

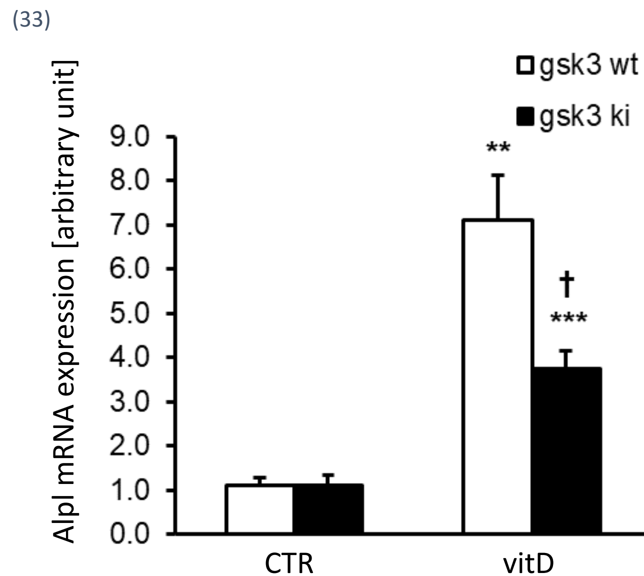

Fig.33. The average value (±) standard error of the mean of (n=6–9; arbitrary units) of osteogenic marker tissue-nonspecific alkaline phosphatase (Alpl) relative mRNA expression in aortic tissue of gsk-3 knock-in mice (ki) and corresponding gsk-3 wild type mice (wt) receiving CTR or high dosed vitD.

($p<0.01$), *($p<0.001$) statistically significant versus CTR gsk-3 wild type mice; †($p<0.05$), statistically significant versus vitD injected gsk-3 wild type mice.

4.2.2 Influence of high-dosed vitamin D on GSK-3α/β phosphorylation at Ser21/9 and β-catenin expression in aortic tissue of mice

Subsequent experiments were conducted to elucidate the relevance of AKT/SGK-resistant Gsk-3α/β during the calcification of the vessels. To this end, western blotting was used to determine expression and phosphorylation levels of Gsk-3α/β in the aortic tissue of vitamin D overloaded mice, as well as beta-catenin expression levels and GPDH as a housekeeping control (Fig.34).

(34)

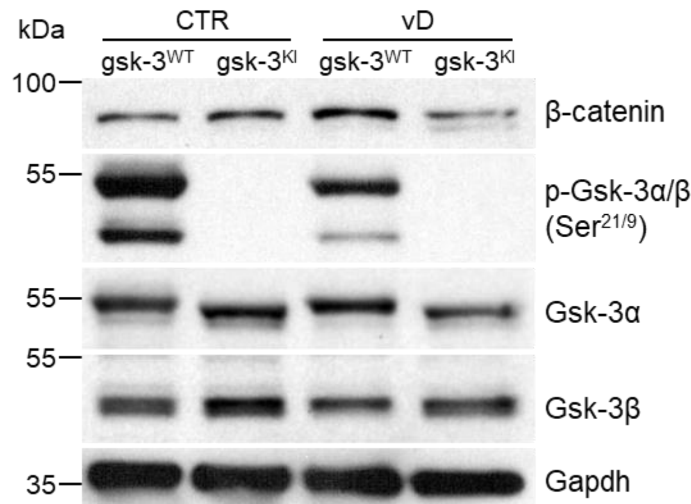

Fig.34. Representative original Western Blots of phosphorylated/total GSK-3α/Gapdh protein as well as beta catenin and Gapdh protein expression in aortic tissue of gsk-3 knock-in mice (ki) and corresponding gsk-3 wild type mice (wt) receiving CTR or high dosed vitD.

As revealed by densiometric analysis of the Western Blots, Vitamin D overload significantly reduced the phosphorylation of Gsk-3α at Ser21 (Fig.35a) and of Gsk-3β at Ser9 (Fig.35b) in the aortic tissue of gsk-3 wild type mice. No expression levels of these phosphorylation sites could be determined in GSK3 ki mice.

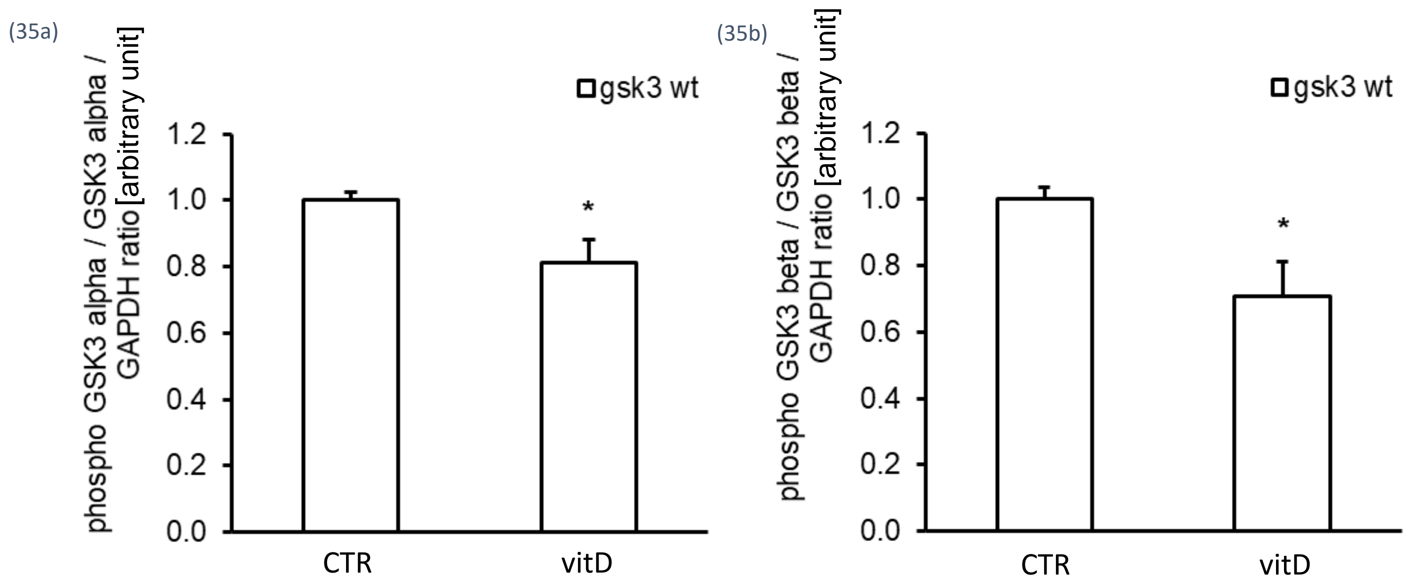

Fig.35. The average value (±) standard error of the mean of (n=4; arbitrary units) normalized phosphorylated/total GSK-3α/Gapdh protein ratio at ser21 (a) and Gsk-3β at Ser9 (b) in aortic tissue of gsk-3 knock-in mice (ki) and corresponding gsk-3 wild type mice (wt) receiving CTR or high dosed vitD.

*($p<0.05$) statistically significant versus CTR gsk-3 wild type mice.

The total Gsk-3α protein levels were not significantly modified in these experiments. However, the protein abundance tended to be higher in control treated GSK3 ki mice as compared to GSK3 wt mice, a difference not reaching statistical significance. Following vitamin D overload, the protein abundance of Gsk-3α tended to be lower in GSK3 ki mice as compared to GSK3 wt mice (Fig.36).

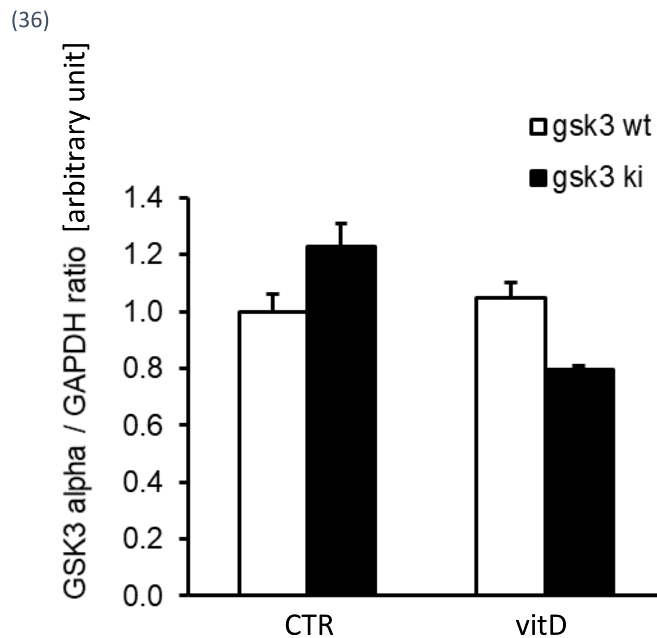

Fig.36. The average value (±) standard error of the mean of (n=4; arbitrary units) total GSK-3α/Gapdh protein ratio in aortic tissue of gsk-3 knock-in mice (ki) and corresponding gsk-3 wild type mice (wt) receiving CTR or high dosed vitD.

Also, the total Gsk-3β protein levels were again not significantly different between the groups of these experiments. Nonetheless, the protein abundance of total Gsk-3β protein tended again to be higher in control treated GSK3 ki mice as compared to GSK3 wt mice, a difference also not reaching statistical significance. Following vitamin D overload, the protein abundance of Gsk-3α tended was not different without an observed tendency in GSK3 ki mice as compared to GSK3 wt mice (Fig.37).

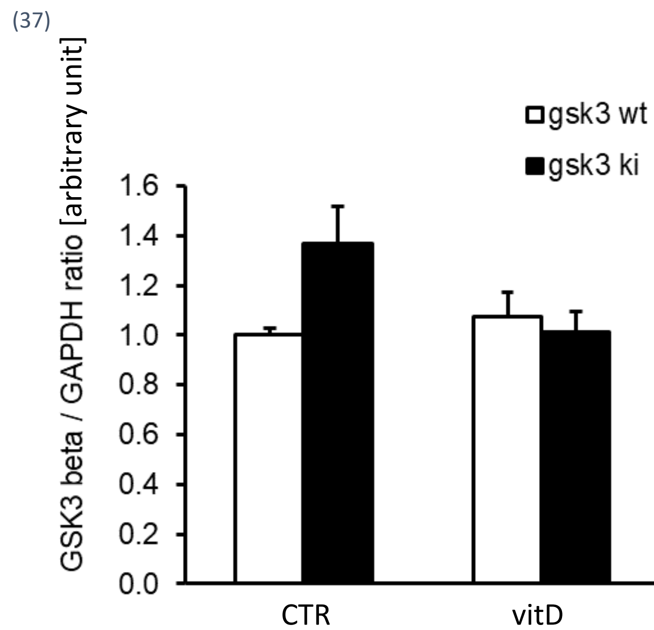

Fig.37. The average value (±) standard error of the mean of (n=4; arbitrary units) total GSK-3β/Gapdh protein ratio in aortic tissue of gsk-3 knock-in mice (ki) and corresponding gsk-3 wild type mice (wt) receiving CTR or high dosed vitD.

Further measurements were performed to investigate the protein abundance of the transcription factor beta-catenin, which has been discussed as regulator of osteo-chondrogenic transdifferentiation. Accordingly, these experiments showed an upregulation of the beta-catenin protein abundance, shown as β-catenin/GAPDH ratio in vitamin D treated GSK3 wt mice. However, this upregulation was virtually absent in GSK3 ki mice after vitamin D overload, and the β-catenin/GAPDH ratio was significantly lower in GSK3 ki mice as compared to GSK3 wt mice (Fig.38).

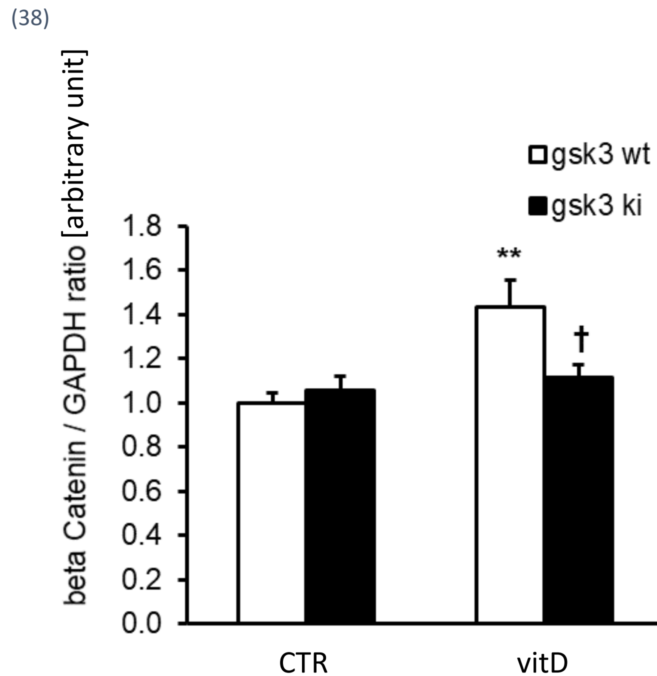

Fig.38. The average value (±) standard error of the mean of (n=4; arbitrary units) of β-catenin/Gapdh protein ratio in aortic tissue of gsk-3 knock-in mice (ki) and corresponding gsk-3 wild type mice (wt) receiving CTR or high dosed vitD.
**(p<0.01) statistically significant versus CTR gsk-3 wild type mice; †(p<0.05) statistically significant versus vitD injected gsk-3 wild type mice.

4.2.3 Role of AKT/SGK-dependent phosphorylation of GSK-3α/β in β-catenin target gene expression during cholecalciferol overload-induced vascular calcification

Further experiments were designed to investigate the putative role of beta-catenin in the phenotype of impaired vascular calcification in GSK3 ki mice. To this end, target genes of beta catenin were measured by RT-PCR. As a first measurement, the mRNA expression of the sodium-dependent phosphate transporter Pit1 was investigated. Vitamin D overload increased expression levels of Pit1 in aortic tissue as compared to control treated mice. The upregulation of mRNA expression of Pit1 was however significantly blunted in GSK3 ki mice (Fig.39).

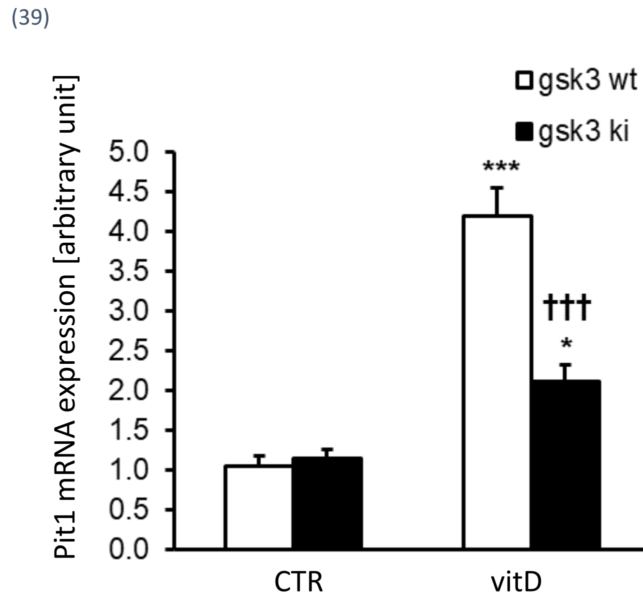

Fig.39. The average value (±) standard error of the mean of (n=6–9; arbitrary units) of Pit1 relative mRNA expression in aortic tissue of gsk-3 knock-in mice (ki) and corresponding gsk-3 wild type mice (wt) receiving CTR or high dosed vitD. *($p<0.05$), ***($p<0.001$) statistically significant versus CTR gsk-3 wild type mice; †††($p<0.001$) statistically significant versus vitD injected gsk-3 wild type mice.

As a further target gene of beta-catenin, Pai1 was investigated. Also, the Pai1 mRNA expression was notably increased in aortic tissues from gsk-3 wt mice. This upregulation was again significantly ameliorated in GSK3 ki mice, and the Pai1 mRNA expression levels were lower in GSK3 ki aortic tissue, as compared to aortic tissue from GSK3 wt mice after vitamin D overload (Fig.40).

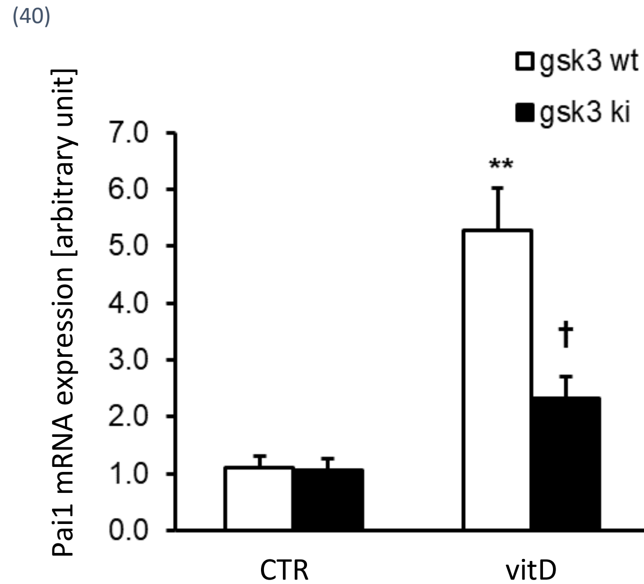

Fig.40. The average value (±) standard error of the mean of (n=6–9; arbitrary units) Pai1 relative mRNA expression in aortic tissue of gsk-3 knock-in mice (ki) and corresponding gsk-3 wild type mice (wt) receiving CTR or high dosed vitD.
**($p<0.01$) statistically significant versus CTR gsk-3 wild type mice; †($p<0.05$) statistically significant versus vitD injected gsk-3 wild type mice.

4.2.4 Role of AKT/SGK-dependent phosphorylation of GSK-3α/β in osteo-/chondrogenic signaling and calcification of aortic ring explants in vitro

Additional experiments were conducted, to confirm the relevance of GSK3 in aortic tissue, independently of systemic effects. To this end, vascular rings were obtained from aortic tissue from GSK3 ki and GSK3 wt mice, and exposed to phosphate in ex-vivo culture.

As a first set of experiments, the calcification response was investigated. Phosphate exposure induced calcification of aortic rings, as determined by increased calcium content. The elevated calcium content following phosphate exposure was significantly reduced in rings from GSK3-ki mice as compared to GSK3 wt mice (Fig.41).

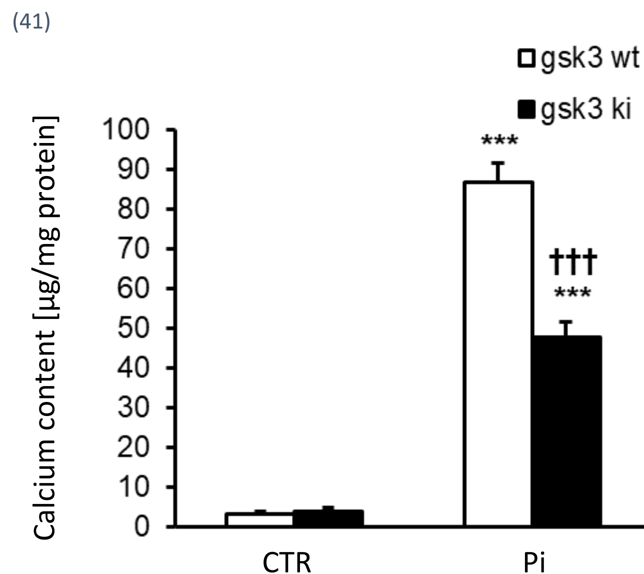

Fig.41. The average value (±) standard error of the mean of (n=6; µg/mg protein) calcium content in the aortic ring explants from gsk-3 knock-in mice (ki) and corresponding gsk-3 wildtype mice (wt) following treatment without (CTR) or with phosphate (Pi) treatment.

***(p<0.001) statistically significant versus CTR gsk-3 wild type aortic ring explants; †††(p<0.001) statistically significant versus phosphate treated gsk-3 wildtype aortic ring explants.

In addition, the mRNA expression of osteogenic marker genes was again determined by RT-PCR. Phosphate exposure strongly upregulated the mRNA expression of msh homeobox 2 (Msx2) mRNA expression in the aortic rings. In accordance with the in-vivo measurements, the upregulation of Msx2 following phosphate exposure was significantly blunted in aortic rings from GSK3 ki mice (Fig.42).

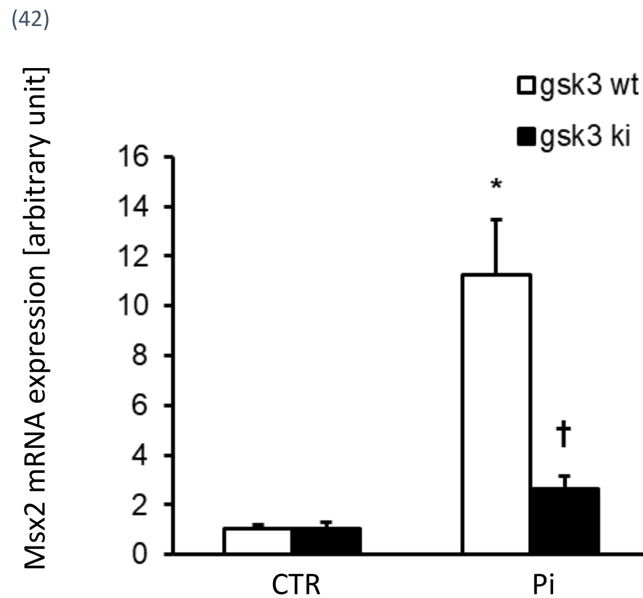

Fig.42. The average value (±) standard error of the mean of (n=6; arbitrary units) of the relative mRNA expression of the osteogenic marker msh homeobox 2 (Msx2) in the aortic ring explants from gsk-3 knock-in mice (ki) and corresponding gsk-3 wildtype mice (wt) following treatment without (CTR) or with phosphate (Pi) treatment.

*($p<0.05$) statistically significant versus CTR gsk-3 wild type aortic ring explants; †($p<0.05$) statistically significant versus phosphate treated gsk-3 wild type aortic ring explants.

As an additional osteogenic marker, the mRNA expression levels of core-binding factor a1 (Cbfa1) were determined. The mRNA expression levels of Cbfa1 were upregulated following phosphate exposure in aortic ring explants from both genotypes. However, the upregulation of Cbfa1 mRNA expression was severely blunted in aortic rings from GSK3 ki mice. Thus, following phosphate exposure, the mRNA expression of Cbfa1 was significantly lower in rings from GSK3 ki mice as compared to rings from GSK3 wt mice (Fig.43).

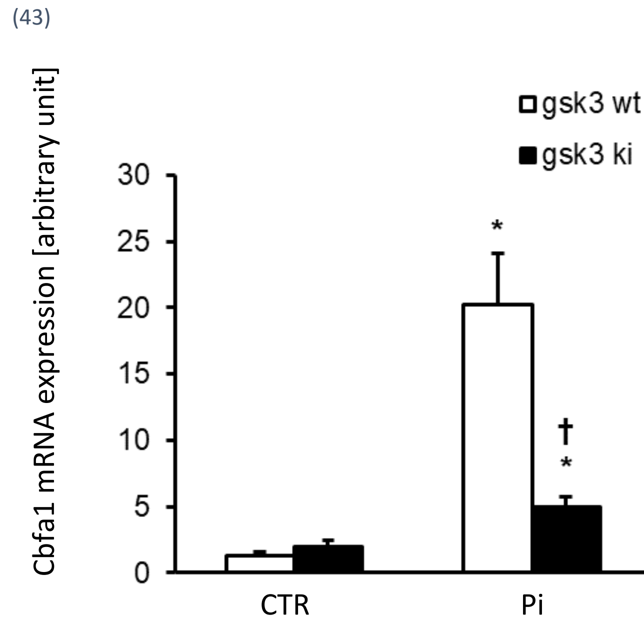

Fig.43. The average value (±) standard error of the mean of (n=6; arbitrary units) of the relative mRNA expression of the osteogenic marker core-binding factor a1 (Cbfa1) in the aortic ring explants from gsk-3 knock-in mice (ki) and corresponding gsk-3 wildtype mice (wt) following treatment without (CTR) or with phosphate (Pi) treatment.

*($p<0.05$) statistically significant versus CTR gsk-3 wild type aortic ring explants; †($p<0.05$) statistically significant versus phosphate treated gsk-3 wild type aortic ring explants.

As a further measurement, the mRNA expression levels of the osteogenic enzyme tissue-nonspecific alkaline phosphatase (Alpl) was determined. Again, the mRNA expression levels of Alpl was increased in aortic ring explants by phosphate exposure. However, similar to previous observations, the mRNA expression of Alpl was significantly lower in aortic ring explants from GSk3 ki mice, as compared to tissues from GSK3 wt mice (Fig.44).

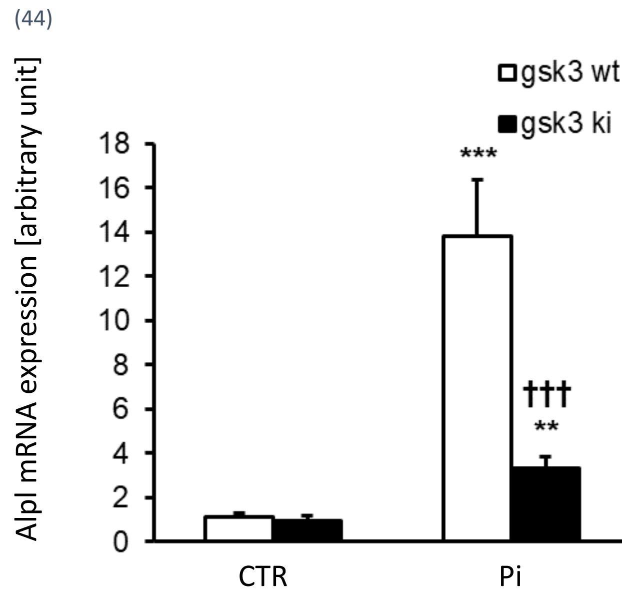

Fig.44. The average value (±) standard error of the mean of (n=6; arbitrary units) of the relative mRNA expression of the osteogenic marker tissue-nonspecific alkaline phosphatase (Alpl) in the aortic ring explants from gsk-3 knock-in mice (ki) and corresponding gsk-3 wildtype mice (wt) following treatment without (CTR) or with phosphate (Pi) treatment.

(p<0.01), *(p<0.001) statistically significant versus CTR gsk-3 wild type aortic ring explants; †††(p<0.001) statistically significant versus phosphate treated gsk-3 wild type aortic ring explants.

5. Discussion

5.1 Vascular calcification

Medial vascular calcification has been considered as a consequence of aging, diabetes and chronic kidney disease.[94, 95] Several severe clinical consequences were associated with this kind of vascular calcification such as; stroke, heart failure, and coronary events, which are associated with increased mortality.[95, 96] Vascular calcification is considered a very common disease in the current days, and it's usually combined with other cardiovascular disorders.[97] The vascular smooth muscle cells are considered to play the central function in causing the calcification of the vessels, by undergoing differentiation to osteoblast-like cells and generating matrix vesicles that serve as a basic structure for calcium–phosphate accumulation in the wall of the vessels.[97, 98] Therefore, the calcification of the vessels is believed to be a complex and highly organized process that includes activating the signaling of the cellular pathways.[99]

The key mechanisms of cellular signaling that induce vascular smooth muscle cells to promote vascular calcification are still not well defined.[99, 100] Therefore, in the course of this work it was attempted to investigate signaling of vascular smooth muscle cells during calcification processes, that are considered to be of important relevance in the onset and progression of vascular calcification.[101] The work focused on the anti-calcific pathways of magnesium and the glycogen synthetase kinase 3 (GSK3). It could be observed, that magnesium is able to inhibit vascular smooth muscle osteogenic reprogramming, and this effect involves activation of the calcium-sensing receptor. Furthermore, it was noted, that phosphorylation and inactivation of GSK3 by SGK/AKT may be a permissive factor to turn vascular smooth muscle cells towards calcification.

5.1.1 Inhibitory effect of magnesium chloride on vascular calcification

In order to evaluate the effects of magnesium on calcification, in-vivo and in-vitro experiments were performed, which confirmed that magnesium chloride is able to prevent the development of vascular calcification. As already hinted by the literature, these experiments revealed that applying magnesium chloride to mice injected by vitamin D, or

phosphate-treated human aortic smooth muscle cells reduces vascular calcification.[101, 102] It has been however unclear, if magnesium may act via a cell-mediated mechanism, or passive interference with calcium-phosphate precipitation.[103]

5.1.2 Magnesium chloride inhibits osteogenic reprogramming of osteogenic factors

In the current set of experiments, the osteogenic reprogramming of vascular smooth muscles cells could be blunted a by magnesium chloride addition, as suggested by other observations.[103] It has been speculated, that the nanoparticles of calcium-phosphate that were accumulated by increasing calcium and/or phosphate concentrations, lead to the osteogenic reprogramming.[104] This is supported by the observation, that pre-formed hydroxyapatite particles were able to induce osteogenic reprogramming, as determined by upregulation of osteogenic markers.[104, 105] The effects of magnesium chloride on calcification were associated with intracellular changes marking trans-differentiation. Furthermore, in calcifying cell culture, magnesium impaired increased alkaline phosphatase activity, a key occurrence in vascular calcification.[106] This suggests that magnesium may mediate direct effects on cells, beyond preventing calcium-phosphate precipitation.

Magnesium was also able to blunt calcification in animal experiments, along with reduced osteogenic transdifferentiation of vascular tissue. Similar observations were made recently in an uremic animal model.[107] Other animal models of calcification also confirmed a protective effect of magnesium treatment on vascular calcification .[108] This is in line with previous observations, that magnesium chloride may be beneficial to prevent the calcification of vessels in patients with chronic kidney disease.[109]

5.1.3 Magnesium chloride and its effect of activating the calcium-sensing receptor

It was already described, that the calcium-sensing receptor activation in vascular smooth muscle cells may inhibit both the calcification and osteo-inductive transformation.[110] Dysfunction of the calcium-sensing receptor in vascular smooth muscle cells is associated with enhanced calcification.[111] By applying magnesium chloride, the calcium-sensing receptor expression was notably upregulated in vascular smooth muscle

cells on protein and mRNA level. Accordingly, the calcium-sensing receptor activity in the parathyroid gland is modulated by magnesium chloride.[112] The effect of magnesium chloride decreases the excretion of parathyroid hormone from parathyroid cells through calcium-sensing receptor activation, but its influence may be weaker than calcium ions.[112]

Furthermore, the blunting influences of magnesium chloride on the calcification of the vascular smooth muscle cells were also replicated by gadolinium, a calcium-sensing receptor agonist.[113] On the other hand, the pharmacological inhibition of calcium-sensing receptor blocked the protective influences of magnesium chloride on the calcification of the vascular smooth muscle. Similar effects were observed when silencing the calcium-sensing receptor.[114]

Accordingly, magnesium chloride may play a decisive role on the calcification of the vessels at least in part by activating the calcium-sensing receptor. Active cellular mechanisms were suggested previously to mediate the effects of magnesium, advocating against solely a physico-chemical inhibition of hydroxyapatite formation.[115] Nevertheless, other mechanisms of action mediating the preventive influence of magnesium chloride on vascular calcification cannot be ruled out by the current experiments. Especially, in contrast to earlier observations, magnesium may yet provide inhibitory effects on hydroxyapatite formation.[116]

However, other cellular effects may be involved in the anti-calcific effects of magnesium, as suggested by the observation, that live cells are needed for the full effect of magnesium on the calcification of vascular smooth muscle cells.[115] Magnesium could be transported by mammalian transient receptor potential melastatin (TRPM) channels.[117] The potential channels considered as magnesium transporters are sensitive to 2-aminoethoxydiphenyl borate.[118] The application of 2-aminoethoxydiphenyl borate inhibits the influences of magnesium chloride on the calcification of the vessels.[119] On other hand, the magnesium chloride influences on calcium-sensing receptor and the transport of magnesium in the cells may interact, in which calcium-sensing receptor may modify the magnesium chloride transport in vascular smooth muscle cells.[120]

Also, other systemic factors modifying vascular calcification may play an important role in the inhibition of calcification by magnesium in-vivo. The current observations cannot rule out such systemic interactions. However, the cell culture experiments already disclose a direct anti-calcific effect of magnesium in vascular smooth muscle cells. This is also supported by the effects of magnesium supplementations in vascular rings (Fig.45, unpublished observations). In these experiments, magnesium supplementation strongly ameliorated the phosphate induced calcification.

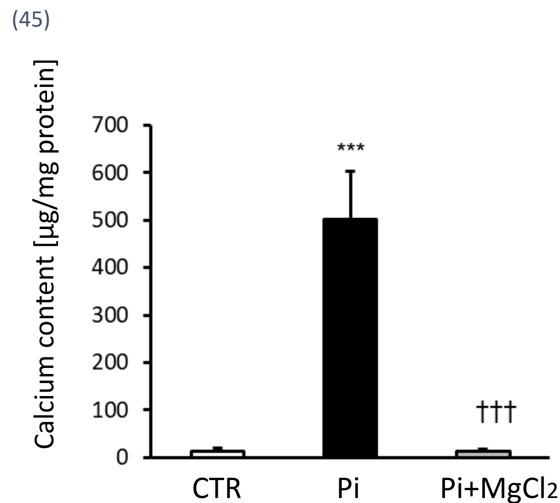

Fig.45. The average value (±) standard error of the mean of (n=7; µg/mg protein) calcium content in aortic ring explants from wildtype mice (wt) following 9 days of treatment without (CTR) or with additional 1.6 mM/l phosphate (Pi) treatment and with the addition of 1.5 mM/l magnesium chloride ($MgCl_2$).
***($p<0.001$) statistically significant versus CTR aortic ring explants; †††($p<0.001$) statistically significant versus phosphate treated aortic ring explants. Unpublished observations.

Therefore, although our observations clearly suggest a contribution of the activation of the calcium-sensing receptor by magnesium to mediate its anti-calcific effects, the extent of the contribution of this observation to inhibition of calcification in patients with calcification still remains to be elucidated.

5.1.4 Interfering with vascular calcification by magnesium in patients with calcification

Especially in patients with chronic kidney diseases, strong calcification of the vessels is observed.[121] The expression of calcium-sensing receptor is decreased in the arterial tissues of the chronic kidney disease patients.[122] By stimulation of the vascular calcium-sensing receptor, the calcification may be ameliorated, as is suggested by animal models. [123]The calcium-sensing receptor of the vascular smooth muscle cells may inherit a necessary function in preventing the vascular calcification and is considered as a possible treatment target for patients with chronic kidney disease with calcified vessels.[124] It was therefore discussed that calcimimetics as stimulators of the calcium-sensing receptors could lead to delay of the onset of vascular calcification.[125]

Therefore, magnesium could play a decisive role in treating vascular calcification for patients suffering from chronic kidney disease. [126] Low magnesium chloride concentrations lead to increased vulnerability to ectopic calcification in patients suffering from chronic kidney disease. [127] Magnesium supplementation may also reduce the risk for developing hypertension. Accordingly, low magnesium diet in rats lead to increased blood pressure and vascular stiffening .[128] Also in the here conducted animal experiments, the magnesium feeding of mice in the Vitamin D treatment decreased the expression of stiffness markers in the aortic tissue.

In conclusion, magnesium is able to reduce calcification in experimental models of vascular calcification. Magnesium may mediate important anti-calcific effects during medial vascular calcification by activation of the calcium-sensing receptor. However, other mechanisms of action for the anti-calcific effects of magnesium cannot be ruled out. Nonetheless, magnesium supplementation may be a feasible treatment to prevent calcification in human chronic kidney disease patients.

5.2 Role of SGK/AKT-dependent inhibition of GSK3 during vascular calcification

5.2.1 GSK3 phosphorylation and vascular calcification

Further experiments were conducted to investigate the cellular signaling pathways of vascular calcification. To this end, the role of the glycogen synthetase kinase 3 (GSK3) in osteo-chondrogenic reprogramming was studied. The experiments in GSK3 knock-in mice suggested, that AKT/SGK-dependent phosphorylation of GSK-3α/β take a part in the signaling regulating vascular osteo-induction and calcification.[90, 129] The impairment of AKT/SGK-dependent phosphorylation of Gsk-3α/β is capable to decrease the osteo-/chondrogenic transdifferentiation and calcification of the vessels in the high-dose vitamin D injected mouse model. This was associated with changes in beta-catenin abundance, a transcription factor inheriting pro-osteogenic effects in vascular smooth muscle cells.[130] Surprisingly, the findings showed that the calcification of the vessels induced by high dosed vitamin D injection was accompanied a decrease of Gsk-3α/β phosphorylation at Ser21/9 in the vascular tissue, the target site of the kinases AKT and SGK.[131] In view of these observations, it could be speculated that the reduction of this inhibitory phosphorylation is an endogenous pathway limiting the onset of calcification.[132]

Calcifying conditions are capable to decrease AKT phosphorylation, but increase SGK1 expression.[133, 134] This tempts to cause speculation, that AKT could be the main controller of Gsk-3α/β phosphorylation during calcifying situations stimulated by dysregulation of mineral homeostasis.[135, 136] According to that, the artificial stimulation of AKT by lack of PTEN induced an increased calcification of the vasculature.[69] Beyond these observations, the role of AKT during vascular calcification is still unclear, as it also may promote effects that could inhibit calcification.[137]

An elevated Gsk-3 phosphorylation is observed during other calcification models.[138] At least in theory, the pattern of decreased Gsk-3α/β phosphorylation could be especially due to the high dosed vitamin D treatment. Previously, it was already noted that the vitamin D mimic paricalcitol is capable to decrease Gsk-3β expression .[139] Furthermore, elevated plasma 25-hydroxyvitamin D3 concentrations are associated with less Gsk-3 phosphorylation in muscular tissues.[140] The high dose vitamin D model is

an artificial model, presumably inducing mineral stress and formation of calcium-phosphate particles by overload of calcium. On the other hand the mineral stress associated with chronic kidney disease patients originates from impaired phosphate excretion by the kidney and is combined with decreased concentrations of active vitamin D.[141] These two situations cause distinct mineral stress and lead to calcification of the vasculature, but the mechanisms may show variations.

However, the interference with AKT/SGK-dependent phosphorylation of Gsk-3α/β during vitamin D high dose is clearly capable to decrease osteo-/chondrogenic trans-differentiation and the calcification of the vasculature. This is supported by the observations, that aortic rings from AKT/SGK-resistant Gsk-3α/β mice are more resistant to phosphate-induced osteo-inductive reprogramming and calcification. The results in aortic ring explants ex vivo show that the effects are not generally related to possible influence of Gsk-3α/β on differences of systemic calcium or phosphate processing.[142] Gsk-3α/β could thus be part of an inhibitory signaling cascade during vascular calcification, that inhibits osteo-chondrogenic reprogramming.

5.2.2 Mechanisms mediating the effects of GSK-3 on vascular calcification

It has been discovered, that GSK-3 is a key controller of the transcription factor β-catenin.[143] GSK-3 is part of a destruction complex, that degrades and therefore inhibits β-catenin.[144] The two isoforms of alpha and beta catenin have been considered as redundant in the regulation of beta-catenin.[144, 145]

The transcription factor β-catenin has been considered as a necessary mediator during the calcification of the vasculature.[146] β-catenin is able to increase the expression of its target genes, which also include the type III sodium-dependent phosphate transporter Pit1 and plasminogen activator inhibitor Pai1.[147, 148] These proteins are known to play a decisive function during osteo-chondrogenic trans-differentiation of vascular smooth muscle cells.[149] Interestingly, β-catenin has also been implicated in atherosclerosis.[150]

The regulation of β-catenin may however not the primary mechanisms, mediating the observed effects in the GSK3 ki mice. It was recently discovered, that two separate

pathways of function exist for GSK3: AKT-GSK-3 and a WNT-GSK-3-β-catenin pathway.[151] Due to differing cellular compartments, these pathways could be separate from each other.[151] However, this may not be completely distinct, as it was shown that AKT can promote WNT– β-catenin-induced alkaline phosphatase activity.[152]

Therefore, it may be that β-catenin contributes to the observed anti-calcific effects in the GSK3 ki mice. However, the regulation of β-catenin in these mice may also be a secondary event, due to other regulations. The exact mechanisms, how the anti-calcific effects in the GSK3 ki mice are mediated, cannot be exactly determined at the current state of knowledge.

6. Conclusion

Vascular calcification is an occurrence of medical importance, and associated with cardiovascular mortality. This calcification is most extensive in patients with chronic kidney disease. The current work attempted to elucidate important signaling pathways of vascular calcification.

Magnesium chloride was described as a very effective way to prevent vascular calcification in cell culture and a mouse model of vascular calcification. It was found that magnesium was able to regulate the calcium-sensing receptor and this receptor was required for the anti-calcific effects of magnesium.

Furthermore, the AKT/SGK-dependent phosphorylation of GSK-3 was shown as an important contributor to the development of vascular calcification. In mice, in which these phosphorylation sites were absent, the extent of vascular calcification was ameliorated. This effect was associated with ameliorated expression of Pit1 and Pai1 during calcification.

These observations extend the knowledge about the signaling pathways of vascular calcification. This understanding may also provide some aid in order to translate new therapeutic treatments to patients with chronic kidney disease, which may reduce vascular calcification and improve cardiovascular mortality.

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8. Declaration of contribution

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The experiments were performed by Rashad Tuffaha, with assistance from I Alesutan and T Auer in the cell culture and analysis experiments, as well as J Voelkl and M Feger in the animal experiments. B Pieske contributed to data analysis and interpretation. F Lang supervised the study.

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