THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

FERRITIN: A POTENT INHIBITOR OF VASCULAR CALCIFICATION AND OSTEOBLAST ACTIVITY

By: Abolfazl Zarjou, MD

Supervisor: József Balla MD, PhD, DSc

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF KÁLMÁN LAKI

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ABBREVIATIONS

ALP - Alkaline phosphatase

ANOVA - Analysis of variance

ApoFt – Apoferritin

Cbfa-1 - Core binding factor alpha-1

CKD - Chronic kidney disease

CO - Carbon monoxide

CO2 – Carbon dioxide

CP – Ceruloplasmin

CT - Computed tomography

DFO – Deferoxamine

DMEM - Ethylenediaminetetraacetic acid

EDTA – Dulbecco's Modified Eagle Medium

ESRD - End stage renal disease

FBS – Fetal bovine serum

Fe – Iron

Ft-H – Heavy subunit of ferritin

Ft-L – Heavy subunit of ferritin

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GM – Growth medium

HCl – Hydrochloric acid

HO-1 - Heme oxygenase-1

HO-2 - Heme oxygenase-2

HRP - horseradish peroxidase

IgG - Immunoglobulin G

mRNA - Messenger RNA

NaCl – Sodium chloride

NaOH – Sodium hydroxide

OB – Osteoblast

PAGE - Polyacrylamide gel electrophoresis

PBS - Phosphate buffered saline

Pi – Inorganic Phosphate

Pit-1 - Type III sodium-dependent phosphate co-transporter

RNA – Ribonucleic acid

SDS - Sodium dodecyl sulfate

siRNA - Small interfering RNA

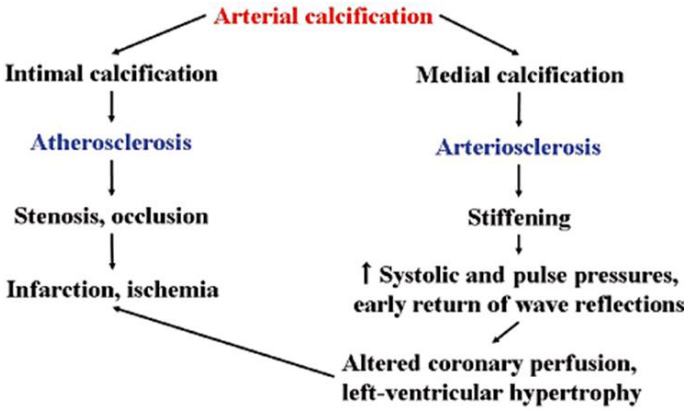
SMC - Smooth muscle cells

SnPP - Tin-Protoporphyrin

INTRODUCTION

Vascular Calcification

Cardiovascular disease is a major cause of morbidity and mortality among patients with chronic kidney disease (CKD) (1, 2). Vascular calcification plays a role in the pathogenesis of atherosclerosis, diabetes and CKD and is an independent risk factor associated with cardiovascular disease and mortality (3). Furthermore, vascular calcification has been associated with cardiovascular complications such as atherosclerotic plaque burden (4, 5), myocardial infarction (6, 7), coronary artery disease (8, 9), postangioplasty dissection (10), and increased ischemic episodes in peripheral vascular disease (10). The first description of vascular calcification was provided by Rudolf Ludwig Karl Virchow, the father of cellular pathology, who noted the presence of stiff, "bone-like" consistency in atheroma in 1863. It is now evident that vascular calcification occurs in 2 layers of arterial wall: tunica intima and tunica media (11, 12). Tunica intima is a layer of endothelial cells supported by internal elastic lamina, while tunica media comprises a smooth muscle layer and elastic tissue. In atherosclerosis, endothelial injury is followed by adhesion of leukocytes as well as maturation of monocytes into macrophages with lipid uptake (11, 12). Smooth muscle cells (SMC) migrate from the media to intima and proliferate. Fatty streaks and fibrous plaques enlarge and bulge into the arterial wall in which calcification causes narrowing of the lumen. Intimal or atherosclerotic calcification is more prevalent in large arteries such as aorta, and occurs more frequently in elderly, hypertensive, dyslipidemic and diabetic patients.



Scheme 1. Simplified consequences of different arterial calcification

Medial calcification was first described in 1903, as a sheet-like calcification in the SMC layer of arterial wall without lipid or cholesterol deposit. The increase in arterial stiffness caused by such medial calcification can result in poor arterial compliance, systolic hypertension, widening of pulse pressure, left ventricular hypertrophy, impaired coronary perfusion and myocardial ischemia (13, 14). These findings, their association with cardiovascular disease and pathophysiological outcomes are summarized in scheme 1. Furthermore, arterial stiffness has been found to be a predictor of mortality in hemodialysis and peritoneal dialysis patients (13, 14).

Medial calcification is particularly common in patients with end stage renal disease (ESRD) and frequently found in peripheral arteries, such as epigastric, femoral, and radial arteries (15-17). Earlier evidence revealed substantially higher prevalence of vascular calcification in young adults on chronic hemodialysis compared to the general population in the same age range, indicating the influence of CKD-related risk factors on the development of vascular calcification (13, 18, 19). In 1979, Ibels et al. studied the pathology of arteries obtained from dialysis patients and discovered an increase in arterial calcification compared with a normal population of the same age (20). In CKD, studies reported prevalence of vascular calcification ranges from 47%-92% (21-23). Vascular calcification has been shown to predict cardiovascular events and mortality in the entire spectrum of patients with CKD as well as in kidney allograft recipients (3, 24). Such overall vascular stiffness due to vascular calcification has a significant negative effect upon survival for patients requiring renal replacement therapy (25-27). For instance, in a well-designed study, quantitative computed tomography (CT) scanning was used to assess the relationships among progressive calcification of the superficial femoral artery, concomitant femoral arterial stiffness, and mortality (27). At two years, progressive calcification was seen in 57% of patients with increases in calcification of the vascular tree correlating with increases in arterial stiffness. Additionally, such vascular

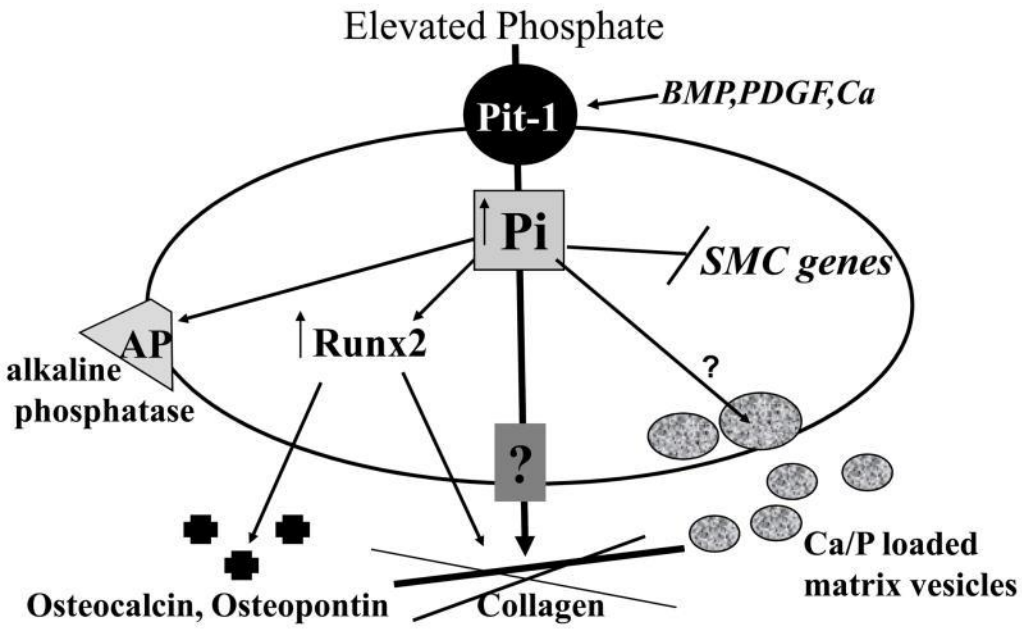
calcification was present in almost all patients who died (95%). In contrast, most patients with an initial calcification score of zero did not develop calcification over this period (27).

In addition to traditional cardiovascular risk factors including aging, smoking, diabetes, dyslipidemia, other novel risk factors such as CKD related risks such as inflammation, and elevated c-reactive protein phosphate (Pi) retention, excessive calcium intake, past dialysis experience, decreased calcification inhibitors, vitamin D deficiency and increased FGF-23 are also associated with the severity and progression of vascular calcification (22, 28-30).

Vascular calcification was in the past considered to be an imminent, passive, degenerative process involving advanced atherosclerotic lesions; however, recent research has revealed numerous similarities with actively controlled processes occurring in the bone tissue (31). Such active and regulated process has created a great deal of interest and research to better understand the pathophysiology of vascular calcification. These studies have revealed that the mechanism of vascular calcification is complex and multifactorial (28, 31). In this regard, increased level of Pi that is a common finding in later stages of CKD has been shown to be a major culprit of transdifferentiation of vascular smooth muscle cells into osteoblast like cells (32, 33). Advanced CKD patients develop hyperphosphatemia due to impaired renal Pi excretion. High serum Pi levels have been shown to have detrimental consequences on cardiovascular homeostasis and might be considered as a ‘vascular toxin’ (33). Scheme 2 is summarizing the effect of elevated Pi on SMC. Note that following increase of intracellular Pi level via Pit-1 transported, Cbfa-1 (also known RUNX-2) is upregulated and will subsequently induce osteoblast specific genes such as collagen type I, osteocalcin, etc. and simultaneously SMC specific genes are downregulated.

Elevated levels of Pi may directly induce vascular injury and indirectly stimulate osteoblastic differentiation through a type III sodium-dependent phosphate co-transporter (Pit-1) (32). Studies suggest that elevated intracellular Pi may directly stimulate SMC to transform into calcifying cells by activating genes associated with osteoblastic functions (32, 34, 35). This

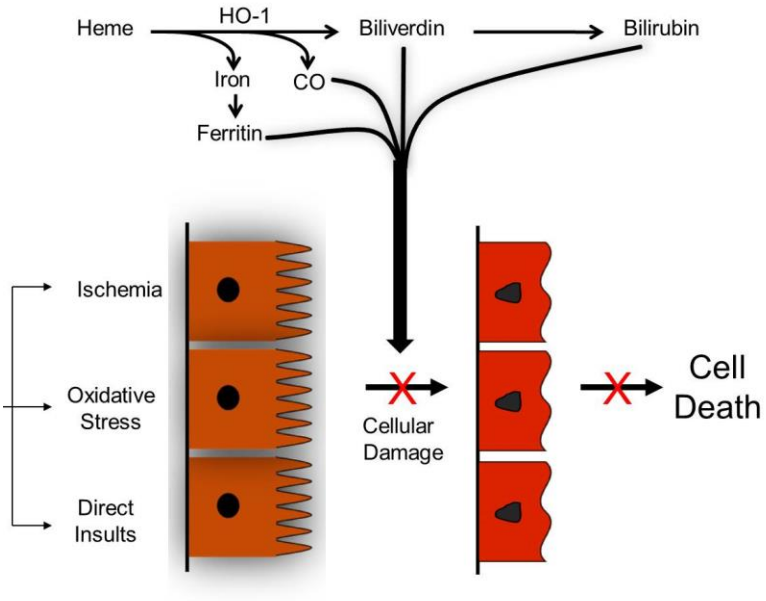
process includes stimulation of a key osteogenic transcription factor, namely core binding factor alpha-1 (Cbfa-1) that is also known as RUNX2. Activation of Cbfa-1 is accompanied by a cascade of signaling pathways that promote differentiation, bone matrix gene expression, and consequently, bone mineralization (32). This entails expression of specific osteoblastic genes such as alkaline phosphatase (ALP) which is an important enzyme in early osteogenesis and osteocalcin, a major noncollagenous protein found in bone matrix that is believed to regulate mineralization and is also known as a pathognomonic feature of osteoblastic terminal differentiation (32). Therefore, based on mounting evidence hyperphosphatemia has been recognized as a major risk factor in patients with severe impairment of renal function.



Scheme 2. Proposed mechanism of high phosphorus (Pi) induced smooth muscle cell transition into osteoblast like cells.

Heme Oxygenase/Ferritin System

The heme oxygenase (HO) was first discovered in the late 1960s to catalyze degradation of heme and generation of bilirubin (36). There are two isoforms of the HO enzyme. While the HO-2 is the constitutive form of the enzyme, HO-1 is the stress inducible isoform of the enzyme that catalyzes the rate-limiting step in heme breakdown resulting in the production of equimolar amounts of biliverdin, iron, and carbon monoxide (CO) (37). Iron subsequently induces the expression of ferritin. Although initially recognized for its role in heme catabolism and erythrocyte turnover, it has become increasingly evident that HO-1, plays an important



Scheme 3. Mechanism of heme oxygenase/ferritin mediated cytoprotection.

protective role in numerous injury settings and clinical conditions (38, 39).

The mechanism underlying this cytoprotective effect involves the ability of HO-1 to catabolize free heme, which is a potent pro-oxidant and prevent it from sensitizing cells to undergo programmed cell death (40). Furthermore, such protective biological activities are conferred via antioxidant, anti-inflammatory, anti-apoptotic and pro-angiogenic properties that have been attributed to CO, biliverdin, bilirubin and ferritin (scheme 3) (38-42). In this regard, tin-protoporphyrin (Snpp) would prevent the HO activity and deferoxamine (DFO) would

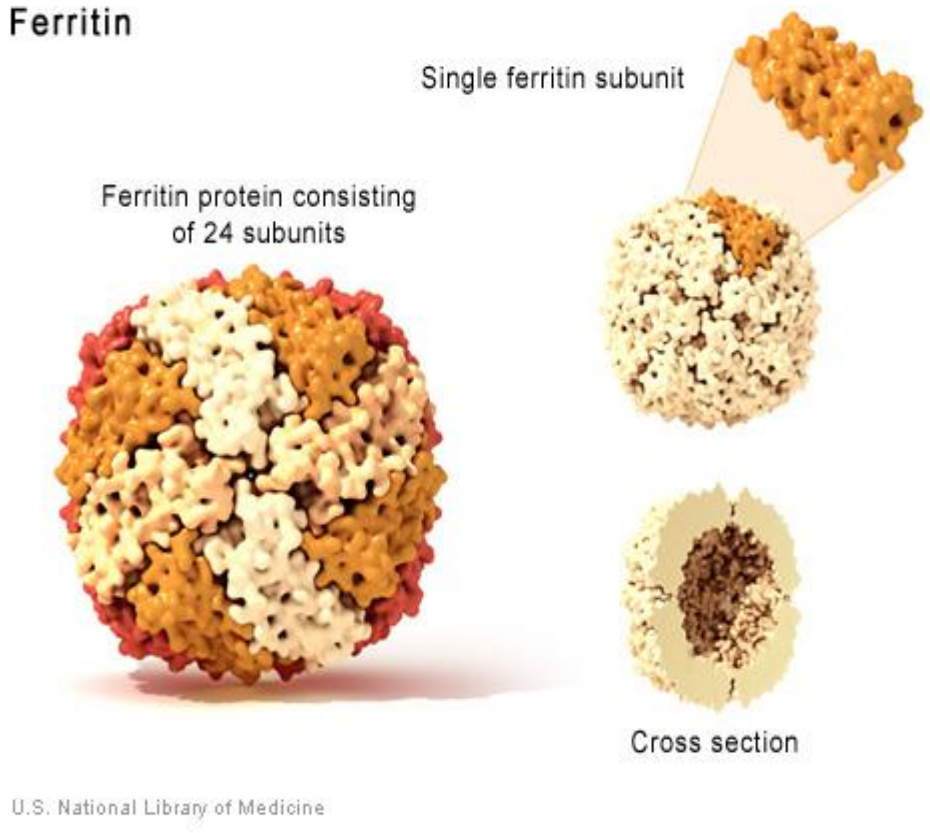
decrease the ferritin induction via sequestration of iron. These findings have been confirmed not only by animal models but also the discovery of two cases of human HO-1 deficiency (43, 44). Heme degradation results in release of iron that in turn induces the expression of ferritin. Iron is essential for almost all living organisms. It participates in a wide variety of fundamental metabolic processes, including oxygen transport, DNA synthesis, and electron transport (45, 46). However, when present in excess, iron poses a threat to cells and tissues. The toxicity of iron is largely based on its ability to catalyze the generation of free radicals, which attack and damage cellular membranes, protein and DNA (45, 46). In fact, iron overload has been associated with pathological conditions including neurodegenerative disorders, liver dysfunction, diabetes and even carcinogenesis among others (47). Therefore, it is apparent that meticulously orchestrated mechanisms to move iron across biological membranes and to ensure that its distribution in multicellular organisms when needed is essential. One such crucial mechanism is the induction of ferritin (scheme 4 depicts the three dimensional structure of ferritin). Ferritin is the most ancient molecule of iron homeostasis with highly conserved three-dimensional structure that carries fundamental functions and new roles arose during evolution (48-50).

As an iron storage protein, ferritin plays a key role in iron metabolism. Its ability to sequester the iron element gives ferritin the dual functions of iron detoxification and iron reserve. The importance of these functions is emphasized by ferritin's ubiquitous distribution among living species (48-50). Ferritin molecules isolated from vertebrates are composed of two types (heavy [H] and light [L] chain) whose proportion depends on the iron status of the cell, the tissue, and the organ (51). In this regard ferritin shell made of H and L chains and the formed shell which is devoid of iron is called the apoferritin.

The H-chain has ferroxidase activity that is essential to prevent cellular damage provoked by reactive oxygen species (52). This site catalyzes the oxidation of Fe(II) with the production of H_2O_2, which is used as an oxidant for further oxidation of Fe(II). Thus, the ferroxidase activity can consume both reagents of the Fenton reaction: H_2O_2 and Fe(II), thereby

minimizing potential injury induced by the activity of the Fenton reaction (53). The L chain has no enzymatic activity but its presence even in small proportions (2-4 chains per shell) accelerates the transfer of iron to the iron core and improves the overall iron sequestering process (54).

Consequently the presence of the two subunit-types enhances the functionality of the molecule. In mammals the H and L chains assemble in the proportion determined by the tissue and the cellular development. The H-rich ferritins are found in heart and brain, have higher ferroxidase activity and have a more pronounced anti-oxidant activity, while the L-rich ferritins of spleen and liver are physically more stable, may contain larger amount of iron and a more pronounced iron storage function (50). The first study that identified ferritin heavy subunit as a potent anti-oxidant was reported by Balla and colleagues (52). Prior to this study, ferritin was regarded as a housekeeper iron storage protein. Numerous studies have since confirmed the



Scheme 4. Three dimensional Structure of Ferritin.

findings reported in Balla et al.'s report (48). Through these studies a distinct portrait has evolved that identifies ferritin as major protective mechanism to prevent oxidative damage. The indispensable nature of FtH has been confirmed in a transgenic mouse model where disruption of FtH by homologous recombination led to early embryonic lethality (55). The protective nature of the HO-1/ferritin system in a diverse range of clinical conditions led us to hypothesize that its induction may play a beneficial role in vascular calcification.

Iron and osteoporosis

Bone is a distinct form of connective tissue that is composed of specialized cells and extracellular matrix. The main feature of bone that distinguishes it from many other forms of connective tissue is the mineralization of its matrix, which produces a rigid tissue capable of not only providing support and posture but also protection of vital organs (56, 57). Osteogenesis is dependent on a specialized cell type, osteoblast that is a secretory cell with the ability of dividing and proliferation. Hence, the degree of bone formation and overall homeostasis is highly dependent on well-functioning osteoblasts. While accumulation of excessive amounts of iron has been linked with various organ dysfunctions, its association with bone loss has only recently been emerging (58-61). Traditionally, decreased levels of sex steroids had been suggested to be major mediator of decreased osteobalstic activity in patients with hemochromatosis.

Emerging evidence however, indicates that iron and its overload have direct deleterious effects on bone, concluding that the effect of iron is to decrease bone deposition rather than to increase bone resorption (59, 61). Despite strong *in vitro* and *in vivo* evidence though, the precise mechanism by which iron can cause a decrease in bone mass and its mineralization was yet to be thoroughly studied. The latter part of this thesis investigated the mechanism by which iron overload causes decreased bone mass.

Our overall hypothesis initially focused on the role of HO-1/ferritin system in the context of vascular calcification. Based on the results of this study and following our observation that induction of this pathway would inhibit vascular calcification via induction of intracellular ferritin, we then asked whether upregulation of ferritin would be responsible for the profound osteoporosis and osteopenia that is commonly observed in patients with hemochromatosis. The following chapters will present detailed findings and conclusions that would support the above hypotheses.

Aims

Part 1

Vascular calcification is a major manifestation or marker of morbidity and mortality. More recent advances have demonstrated that the process of vascular calcification is not a mere passive process as previously thought. In fact it involves transdifferentiation of SMC into osteoblasts that is accompanied by expression of bone specific genes followed by deposition of hydroxyapatite minerals. Patients with ESRD are at increased risk of developing vascular calcification irrespective of age and other comorbidities. Despite improved knowledge of pathomechanisms leading to vascular calcification, therapeutic and/or preventive measures have remained elusive.

HO-1/ferritin system has been shown to be protective in many models of injury and clinical conditions including cardiovascular diseases. However, little was known as to whether activation of HO-1/ferritin system plays any beneficial role in mitigating vascular calcification.

Aim 1:

- Study the effect of HO-1/ferritin induction in high Pi induced SMC calcification
- Identify mechanism of inhibition of vascular calcification by ferritin

Part 2

Iron overload has been associated with osteoporosis and osteopenia. Despite such association the exact role of iron in decreased bone density was unknown. Based on the results of the first part of study we were prompted to investigate the mechanism by which iron may lead to decreased bone mass.

Aim 2:

- Investigate the pathological role of iron overload in decreased osteoblast activity
- Examine the role of ferritin in iron induced inhibition of osteoblast activity and gene expression

MATERIALS AND METHODS

Cell culture and reagents

SMC were purchased from Cambrex, FBS from Gibco, biliverdin was obtained from MP Biomedicals, tin-Protoporphyrin (SnPP) from Frontier Scientific, 1% CO gas from Linde gas and the gas chamber from Billups-Rothenburg Inc. Unless otherwise mentioned all other reagents were obtained from Sigma. Cell cultures were maintained in growth medium (GM) DMEM (high glucose) containing 15% FBS, 100 U/ml penicillin, 100 µg/mL streptomycin and neomycin, and 1 mM of sodium pyruvate. Cells were grown to confluence and used from passages 4 to 8. Iron was introduced as ammonium ferric citrate or ferric sulfate as well as ferrous form. To keep the ferrous state the medium were supplemented with 200 µmol/L of Ascorbic acid. Iron was dissolved in deionidized water. Small interfering RNA (siRNA) specific to HO-1 and negative control siRNA were obtained from Ambion and were transfected with Oligofectamine Reagent (Invitrogen) 24 hours prior to the experiment. Heme, biliverdin and bilirubin were dissolved in NaOH. Final concentration of NaOH was kept below 2 mmol/L in all experiments. This amount of NaOH caused a little change in the pH of the medium (7.40 vs. 7.46) which did not influence calcification, and underlying gene expression of SMC.

Induction of calcification in SMC and OB

At confluence, SMC were switched to calcification medium, which was prepared by adding 4 mmol/L of inorganic phosphate or 2.5 mmol/L for OB to the growth medium. Both growth medium and calcification medium were changed every 2 days. For time-course experiments, the first day of culture in calcification medium was defined as day 0.

Quantification of calcium deposition

Cells grown on 48-well plates were washed twice with PBS and decalcified with 0.6 mol/L HCl for 24 hours at 37^{o}C. Calcium content of the supernatants was determined by the QuantiChrome Calcium Assay Kit (Gentaur). After decalcification, cells were solubilized with a solution of NaOH 0.1 mol/L and SDS 0.1% and protein content of samples were measured with BCA protein assay kit (Pierce). Calcium content of the cells was normalized to protein content and expressed as µg/mg protein.

Mineralization was determined by Von Kossa staining or Alizarin Red. Briefly, for Von Kossa staining he cells were fixed for 15 minutes in ice cold ethanol, and after washing they were incubated with 5% silver nitrate for 15 to 30 minutes followed by 15 minutes of incubation under UV light. For Alizarin Red cells were fixed with ice cold ethanol for 10-15 minutes, washed x2 with PBS (pH adjusted to 4.2) 1-2 times. Filtered 2% Alizarin Red Solution was then added to to the fixed cells and incubated for 10-20 minutes. Cells were then washed with regular PBS and calcium deposition was evaluated under microscope.

Alkaline phosphatase activity assay

Cells grown on 6-well plates were washed with PBS twice, solubilized with 1% Triton X-100 in 0.9% NaCl and assayed for ALP activity. Briefly, 130 µl of Alkaline Phosphatase Yellow Liquid Substrate (Sigma) was combined with 50 µg of protein samples, incubated at 37^{o}C for 30 min, and then the kinetics of p-nitrophenol formation was followed for 30 min at 405 nm using spectrophotometer. Maximum slope of the kinetic curves was used for calculation.

Heme oxygenase enzyme activity assay

Cells grown on P100 dishes were washed twice with HBSS, scraped and centrifuged at 2000 x g for 15 min at 4^{o}C. Cells were resuspended in 300 µl potassium-phosphate (100 mmol/L, pH 7.4) buffer containing 2 mmol/L MgCl2, frozen and thawed three times, sonicated and

centrifuged at 18,000 x g for 10 minutes at 4oC. The supernatant containing cell microsomes was used to measure HO activity as described previously (52). HO activity is expressed as pmol bilirubin formed/mg cell protein/60 min.

Western blot to detect HO-1, ferritin H-, and L-chain

To evaluate HO-1 protein expression cell lysate was electrophoresed in 12.5% SDS-PAGE. For ferritin H- and L-chain detection cell lysate was subjected to 8% non-denaturing PAGE. Western Blotting was performed with a polyclonal anti-HO-1 antibody at 1:2500 dilution (Calbiochem) or with mouse anti-human ferritin H- or L-chain antibodies (from P. Arosio) at 1:1000 dilution followed by HRP-labelled anti mouse IgG antibody. Antigen-antibody complexes were visualized with the horseradish peroxidase chemiluminescence system (Amersham Biosciences). After detection membranes were stripped and reprobed for GAPDH.

Quantification of ferritin and osteocalcin

Ferritin content of cell lysate was measured with the IMx ferritin enzyme immunoassay (Abbott Laboratories). For osteocalcin detection extracellular matrix of cells grown on 6-well plates was dissolved in 300 µL of EDTA (0.5 mol/L, pH 6.9). Osteocalcin content of the EDTA-solubilized extracellular matrix samples was quantified by an enzyme-linked immunoabsorbent assay (Bender MedSystems).

Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated, reverse transcribed and HO-1 mRNA was determined as described previously (62). To measure mRNA levels the 25 µL reaction mixture contained 5 µl of reverse transcribed sample, 0.3 nmol/L of primers and 12.5 µl of iQ SYBR Green Supermix (Bio-Rad) were used. PCRs were carried out using the iCycler iQ Real Time PCR System (Bio-Rad). Results were normalized by GAPDH mRNA levels.

Cbfa-1	F: 5'-CAGGCAGGCACAGTCTTC-3' R: 5'-CAGAGGTGGCAGTGTCATC-3'
HO-1	F: 5'-GGTGATAGAAGAGGCCAAGACTG-3' R: 5'GGTGTCATGGGTCAGCAGCT-3'
HO-2	F: 5'-GCAATGTCAGCGGAAGTGGAA-3' R: 5'-AAGTCACCTGAGGTGGTAGTT-3'

Ferritins and ceruloplasmin

Apoferritin and ceruloplasmin were purchased from Sigma. Human recombinant wild type H chain and L chain ferritins and the H-chain mutant 222 deleted ferroxidase activity were expressed in *E. coli* and purified as described previously (63). Final concentrations of ferritins were 2 mg/mL, which correspond to 4.5 µmol/L for Apoferritin, 3.95 µmol/L for H-ferritin, 4.19 µmol/L for L-ferritin. Final concentration of ceruloplasmin was 4 mg/mL which corresponds to 32.7 µmol/L.

Phosphate measurement

Pi content of the cell lysate was determined by the QuantiChrome phosphate Assay Kit (Gentaur). After 24 hours incubation, cells were washed twice with PBS and solubilized with 1 % Triton and the cell lysates were assayed for Pi. Phosphate content of the cells was normalized to protein content and expressed as µm/L/mg cell protein.

Carbon monoxide exposure

CO at a concentration of 1% (10,000 parts per million; ppm) in compressed air was mixed with compressed air containing 5% CO2 before being delivered into the culture incubator, yielding a final concentration of 400 ppm CO. The incubator was humidified and maintained at 37°C. A CO

analyzer was used to determine CO levels in the chamber. After the chamber had stabilized, no oscillations were measured in the CO concentration.

Osteoblast cell culture and reagents

Human osteoblasts (OB) were purchased from Cambrex and FBS from Invitrogen. Unless otherwise mentioned all other reagents were obtained from Sigma. Cell cultures were maintained in growth medium (GM) DMEM (high glucose) containing 15% FBS, 100 U/ml penicillin, 100 µg/mL streptomycin and neomycin, and 1 mM of sodium pyruvate. Cells were grown to confluence and used from passages 3 to 7. Iron was introduced as ammonium ferric citrate dissolved in deionidized water. Apoferritin and ceruloplasmin were from Sigma. Human recombinant wild type H chain ferritins and the H-chain mutant 222 deleted ferroxidase activity were expressed in *E. coli* and purified as described previously (63). Final concentrations of ferritins were 2 mg/mL and ceruloplasmin was 3 mg/mL.

Statistical analysis

Data are shown as mean ± SD. Statistical analysis was performed by ANOVA test followed by post hoc, Newmann-Keuls test for multiple comparisons. A value of $P < 0.05$ was considered significant and marked with one asterisk, and $P < 0.01$ was considered highly significant and marked with two asterisks.

RESULTS

Heme decreases SMC calcification in a dose responsive manner

To develop an *in vitro* model we cultured SMC in calcification medium. Granular deposits developed in SMC grown in calcification medium for 9 days (Fig 1A, panel II), but not in the control culture grown in normal growth medium (Fig 1A, panel I). Intriguingly, we found that

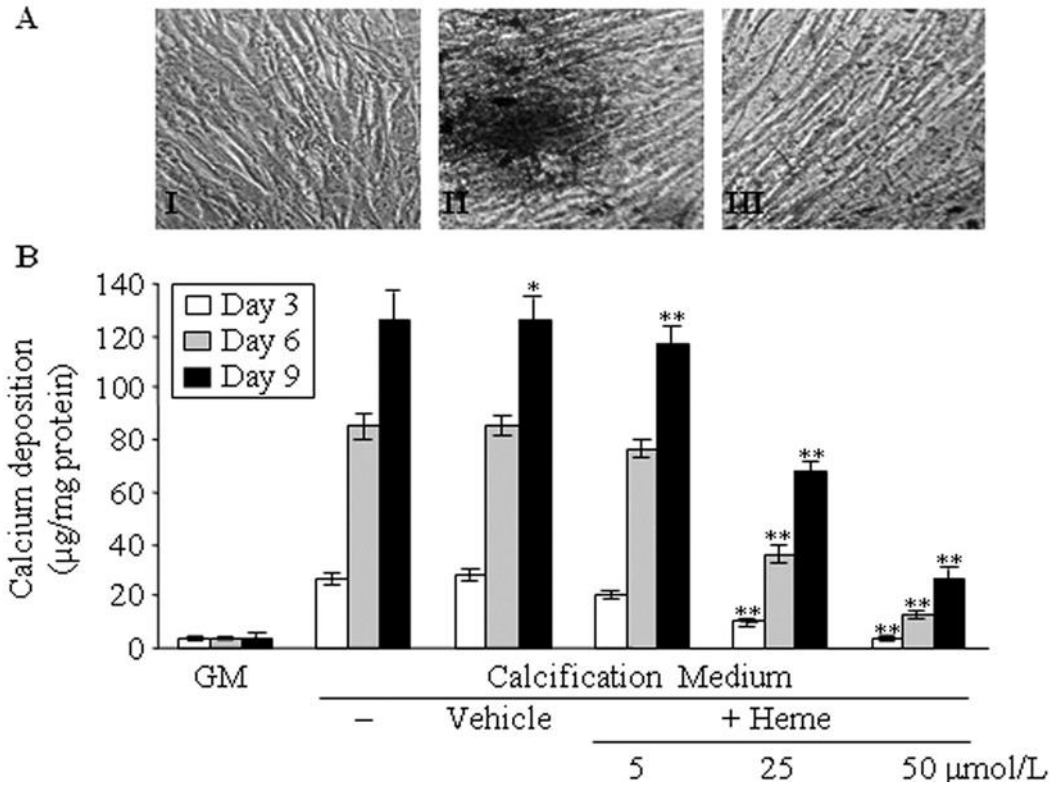


Figure 1. Heme inhibits SMC calcification induced by elevated Pi in a dosage-dependent manner. (A) SMCs were cultured in GM (I) or in calcification medium in the absence (II) or presence of heme (50 μmol/L; III) for 9 d. Von Kossa staining of cells was performed as described in the Methods. (B) SMCs were cultured in GM or in calcification medium alone or supplemented with NaOH (Vehicle, 1 mmol/L) or 5, 25, and 50 μmol/L heme. Calcium contents of cells were measured after 3 (□), 6 (▨), and 9 d (▪) of culture and were normalized by protein content. Data are means ± SD of three independent experiments.

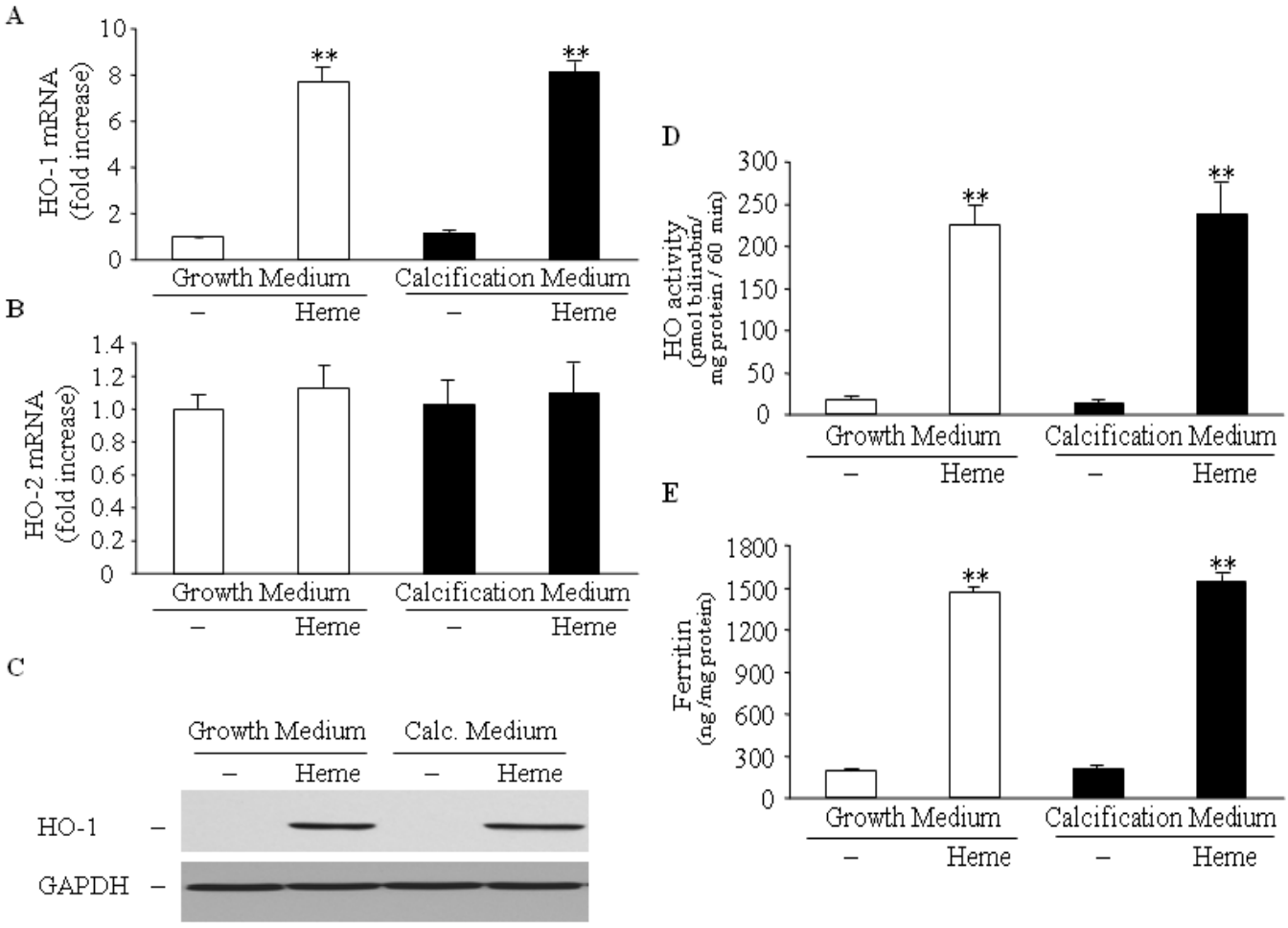


Figure 2. Heme induces HO-1 and ferritin in SMCs. SMCs were cultured in GM or in calcification medium in the absence or presence of heme (50 μmol/L) for 24 h. (A through E) HO-1 mRNA levels (A), HO-2 mRNA levels (B), HO-1 protein expression (C), HO activity (D), and ferritin expression (E) were measured as described in the Concise Methods section. Western blot was stripped and probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and shown as a representative of three experiments. Data are means ± SD of three to five independent experiments each performed in duplicate. $^{**}P < 0.01$.

Extracellular calcium measurements showed that elevated phosphate-induced calcification is time dependent and that the inhibitory effect of heme on extracellular calcification is dose dependent, with a highly significant ($p < 0.01$) suppression at a dose of 25 μmol/L (Fig 1B).

Heme is a strong inducer of HO-1, and as expected we found that HO-1 mRNA, protein and HO activity were elevated in the cells cultured in heme containing medium. Pi level of the medium did not affect this heme-mediated induction of HO-1 (Fig 2A, C, D). In addition we found that heme did not significantly alter HO-2 expression (Fig. 2 B) and it induced expression of ferritin regardless of Pi level of the medium (Fig 2E).

Preliminary studies were performed using heme as a control where cells were only incubated with heme in growth medium for a period of 9 days and such treatment did not affect calcification. The following has been added to the results section: “Heme was also used as a control for a period of seven days and heme alone in the absence of calcification medium did not cause any calcium deposition (data not shown).

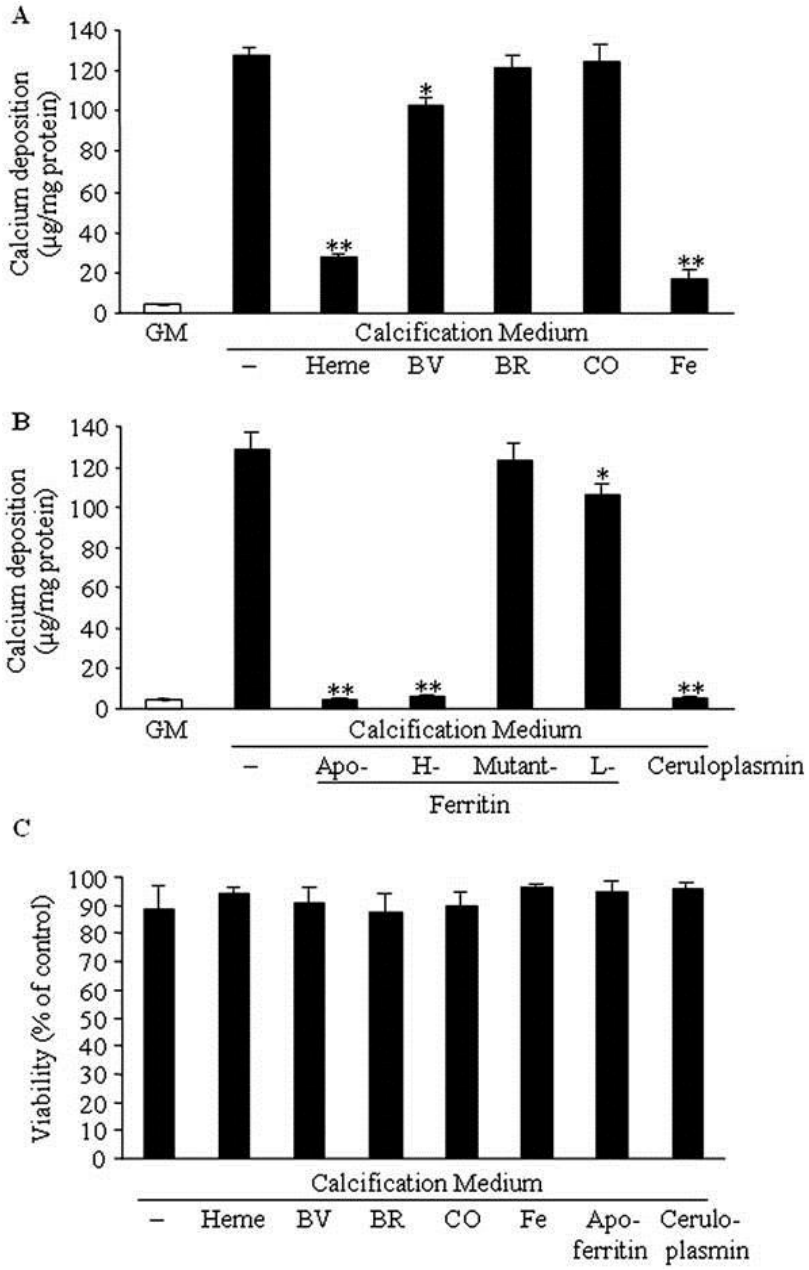


Figure 3. Ferritin/ferroxidase activity is responsible for the inhibition of phosphate-induced SMC calcification. (A) SMCs were cultured in GM or in calcification medium alone or in the presence of heme (50 μmol/L), biliverdin (BV; 50 μmol/L), bilirubin (BR; 50 μmol/L), CO (1%), or iron (50 μmol/L) for 9 d. Calcium content of cells was measured and normalized by cellular protein content. (B) SMCs were cultured in calcification medium alone or supplemented with apoferritin (2 mg/ml), H-ferritin (2 mg/ml), mutant 222 ferritin (2 mg/ml), ceruloplasmin (4 mg/ml), or L-ferritin (2 mg/ml). After 9 d, calcium deposition was measured as described in the Concise Methods section. Graphs show means ± SD of three separate experiments. *$P < 0.05$; **$P < 0.01$. (C) For investigation of whether any of the compounds cause significant toxicity, an MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium-bromide] assay was performed after 9 d of incubation. Data are means _ SD of five separate experiments. **$P > 0.01$.

Ferritin and ferroxidase activity attenuate SMC calcification

Heme induces HO-1 and ferritin, thus it was of interest to analyze which of the two had a major effect on calcification. We also analyzed the role of the end products of HO catalyzed heme degradation by adding them exogenously to the calcification medium. We found that iron, regardless of its ferric or ferrous state (50 μmol/L) completely inhibits calcification (Ferrous state data not shown). Biliverdin at the concentration of 50 μmol/L provided a little but significant ($p < 0.05$) decrease in calcification (Fig 3A). Addition of CO (1%) or bilirubin (50 μmol/L) did not influence calcification (Fig 3A). On the other hand, addition of apoferritin (2 mg/ml) or recombinant H-chain ferritin to the calcification medium abolished calcification (Fig 3B). These two ferritin types have ferroxidase activity, thus we tested another protein with ferroxidase activity, ceruloplasmin. Ceruloplasmin was found to mimic the effect of ferritins at a concentration of 4 mg/ml. The protective effect of L ferritin was minor compared with that of H-ferritin and ceruplasmin. This may have the following explanation. The L- ferritin chains taken up by the cells may coassemble with the endogenous ferritin and thus expand the pool of active ferritins. The H-mutant 222 ferritin which lacks both ferroxidase activity and iron storing capability was not protective at all against mineralization of SMC.

In order to confirm the protective role of ferritin, we inhibited HO using SnPP, a well-known inhibitor of HO activity, and also transfected the cells with small interfering RNA (siRNA) specific for HO-1. We confirmed the efficiency of siRNA and observed around 70% decrease of HO-1 protein expression for up to 4 days post transfection (Fig 4D). In fact, cells treated with heme in the presence of SnPP or siRNA showed very low HO enzyme activity (Fig 4B). Treatment with SnPP or siRNA did not affect the heme-mediated ferritin induction (Fig 4C) and, more importantly, did not influence heme mediated inhibition of calcification (Fig 4A) indicating the paramount role of ferritin in this protection.

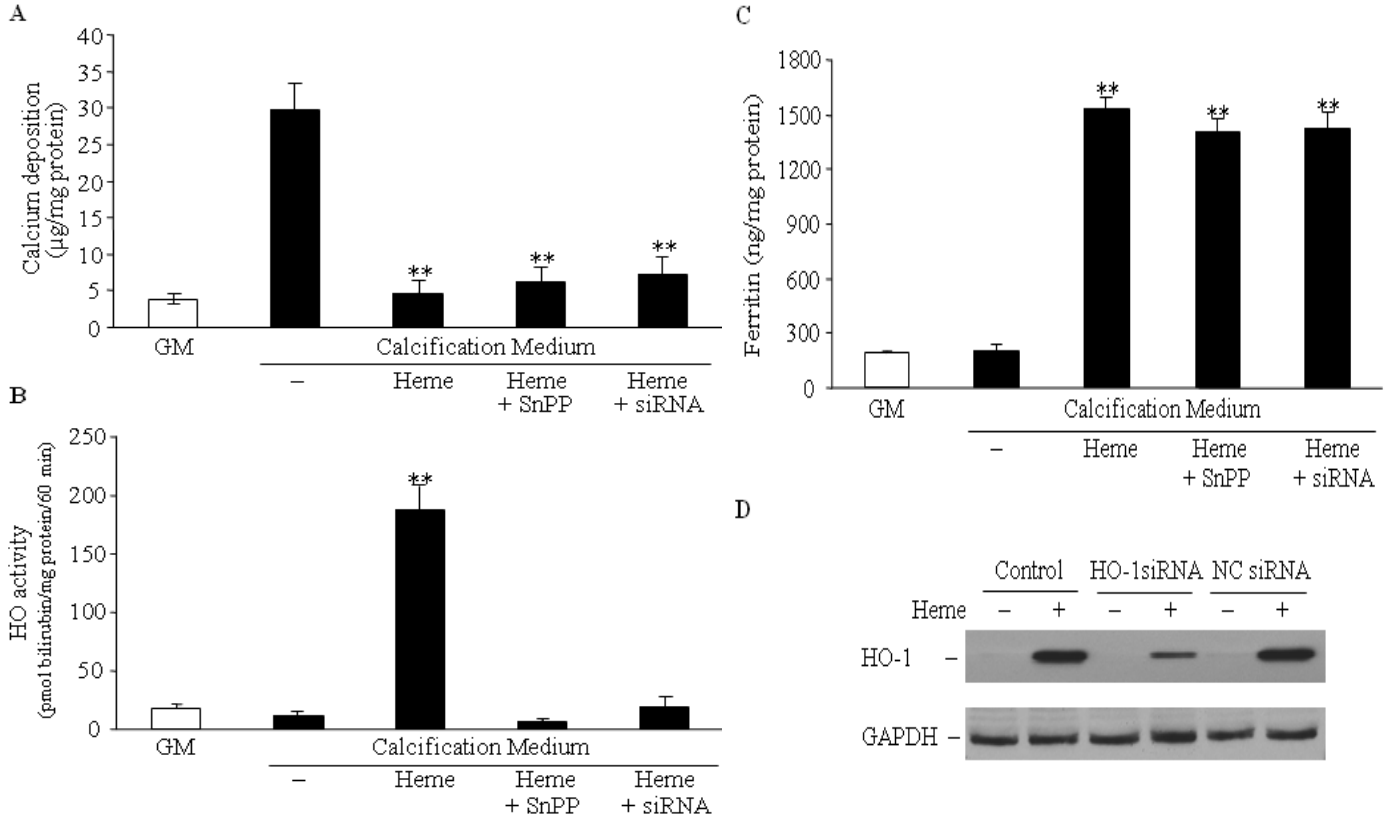


Figure 4. Ferritin induced by heme mediates the inhibition of phosphate-provoked SMC calcification. When applied, cells were transfected with siRNA for HO-1 or negative control siRNA (NC) 24 h before the experiment. SMCs were cultured in calcification medium in the presence of heme (50 μmol/L) or heme and SnPP (50 μmol/L each) for 4 d. (A through C) Calcium deposition (A), HO enzyme activity (B), and ferritin expression (C) were measured. (D) A typical Western blot shows efficacy of HO-1 knockdown by siRNA. Four days after transfection, cells were treated with heme (50 μmol/L) for 24 h, and level of HO-1 protein was determined. Data are means ± SD of three independent experiments each performed in duplicate. $^{**}P < 0.01$.

To further confirm the function of ferritin in the heme or iron induced inhibition of calcification, we selectively downregulated heme-, or iron-induced ferritin synthesis by the iron chelator, deferoxamine (DFO). Treatment of the cells with DFO together with equimolar amount of heme or Fe resulted in a complete block of heme-, or Fe-induced ferritin synthesis of both H- and L-chains as shown by western blot (Fig 5B).

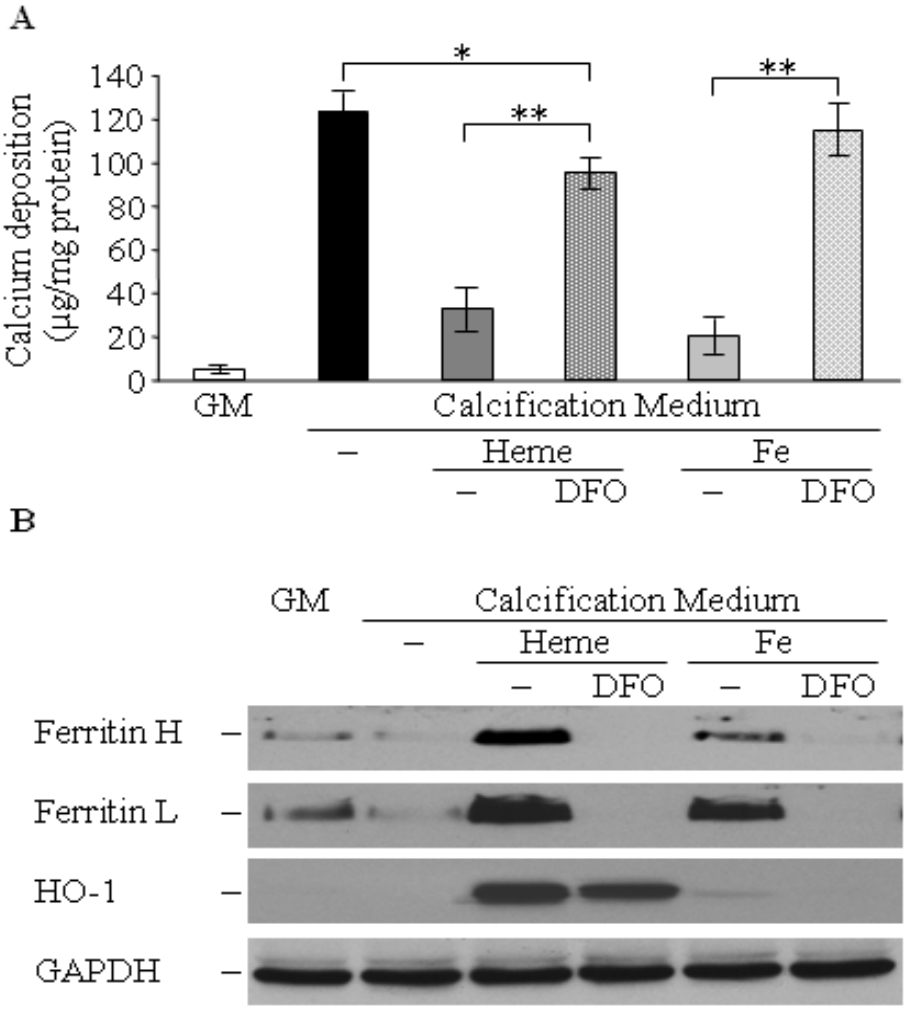


Figure 5. Downregulation of ferritin synthesis by DFO decreased heme- or iron-mediated inhibition of calcium deposition. (A) SMCs were cultured in GM or in calcification medium alone or in the presence of heme (50 μmol/L) or iron (50 μmol/L), with or without equimolar amount of DFO for 9 d. Calcium content of cells was measured and normalized by cellular protein content. (B) Representative Western blots show expression of ferritin H- and L-chains, HO-1, and GAPDH of cells treated as described in A. Data are means ± SD of three separate experiments. **P* < 0.05; ***P* < 0.01.

Downregulation of ferritin synthesis by DFO lead to complete loss of inhibition of calcification by iron (Fig 5A). Moreover, co-treatment with heme and DFO resulted in downregulation of both chains of ferritin, but not of HO-1 (Fig 5B), that was accompanied by substantial decrease in inhibition of calcification (Fig 5A). Mild, but significant ($p < 0.05$) inhibition of calcification was noted which may be attributed to biliverdin derived from HO mediated heme degradation.

Ferritin inhibits osteoblastic differentiation of SMC

It has been shown that vascular calcification in vivo shares similarities with bone mineralization, therefore we asked whether ferritin and its ferroxidase activity solely inhibits mineralization or does it suppress the phenotype transition of SMC into osteoblast-like cells. We examined the activity of alkaline phosphatase (Fig 6) an important enzyme in early mineralization. SMC maintained in calcification medium for 9 days showed around a 7-fold increase in ALP activity compared to control. Supplementation with heme provided a decrease in ALP activity. Similarly, exposures of cells to iron (50 μmol/L) abolished high Pi induced ALP activity. Biliverdin (50 μmol/L) caused some inhibition ($p < 0.05$), while other end-products of HO mediated heme degradation – bilirubin (50 □mol/L) and CO (1%) – failed to decrease ALP activity. Co-treatment of the cells with heme and SnPP demonstrated similar changes in ALP activity as heme alone; on the other hand co-treatment with heme and DFO did not affect the increased ALP activity. Importantly, apoferritin, H ferritin and ceruloplasmin also decreased the activity of ALP to the level seen in controls but the H mutant 222 ferritin was totally ineffective.

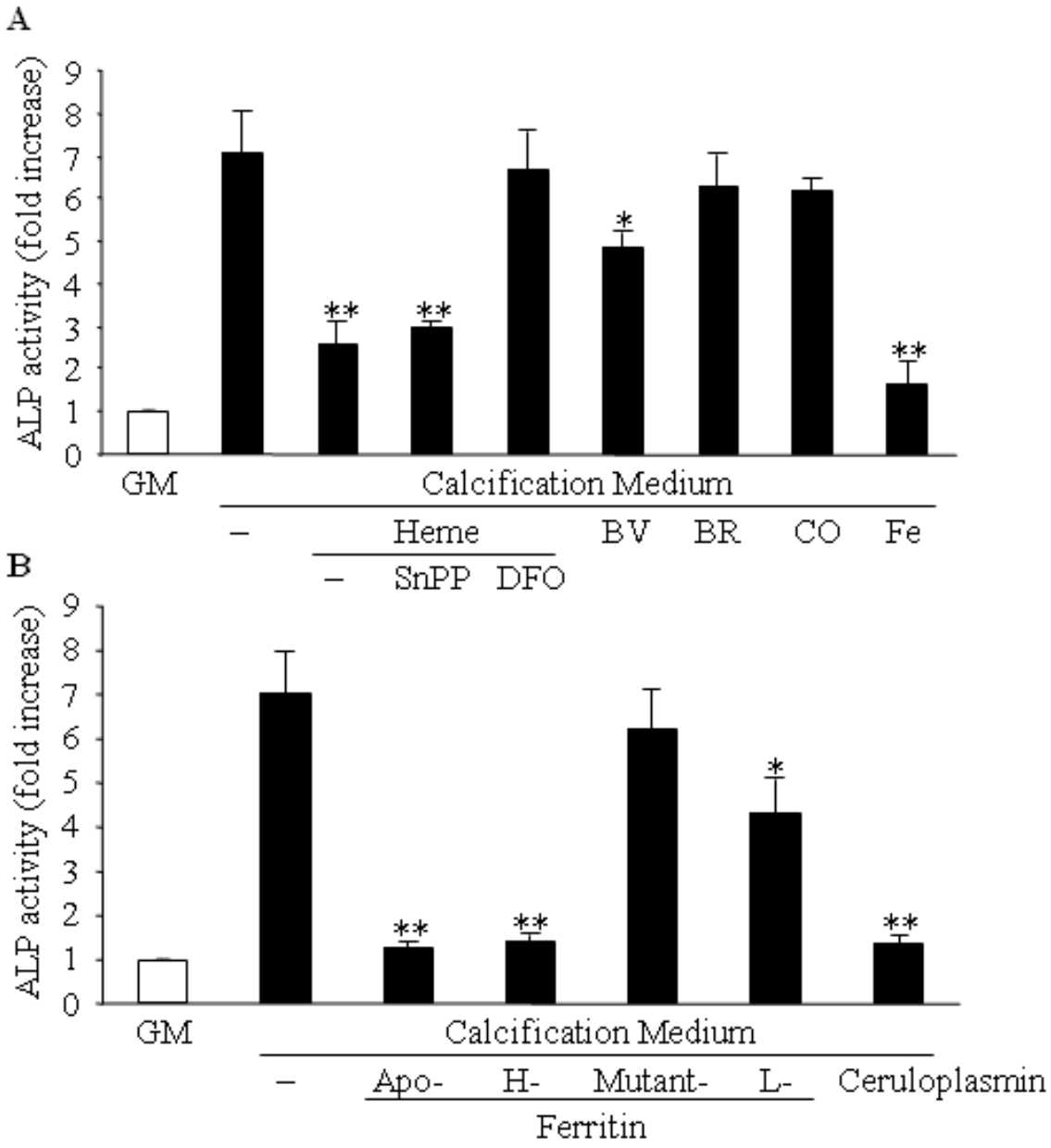


Figure 6. Ferritin attenuates ALP activity induced by elevated Pi. (A) SMCs were cultured in GM or in calcification medium alone or in the presence of heme (50 μmol/L), heme + SnPP (50 μmol/L each), heme + DFO (50 μmol/L each), biliverdin (BV; 50 μmol/L), bilirubin (BR; 50 μmol/L), CO (1%), or iron (50 μmol/L) for 9 d. (B) SMCs were cultured in calcification medium alone or supplemented with apoferritin (2 mg/ml), H-ferritin (2 mg/ml), mutant 222 ferritin (2 mg/ml), ceruloplasmin (4 mg/ml), or L-ferritin (2 mg/ml) for 9 d. ALP activity of cells was measured as described in the Concise Methods section. Data are means ± SD of five independent experiments each performed in duplicate. *$P < 0.05$; **$P < 0.01$.

Hereafter, we investigated the presence of another bone specific protein, osteocalcin in the extracellular matrix. Maintaining of SMCs in calcification medium for 9 days resulted in more than 10-fold increase in osteocalcin content compared to control (Fig 7A). Heme decreased upregulation of osteocalcin, and SnPP did not alter this effect. In contrast, co-treatment of the cells with heme and DFO lead to the loss of osteocalcin downregulation by heme. Iron inhibited

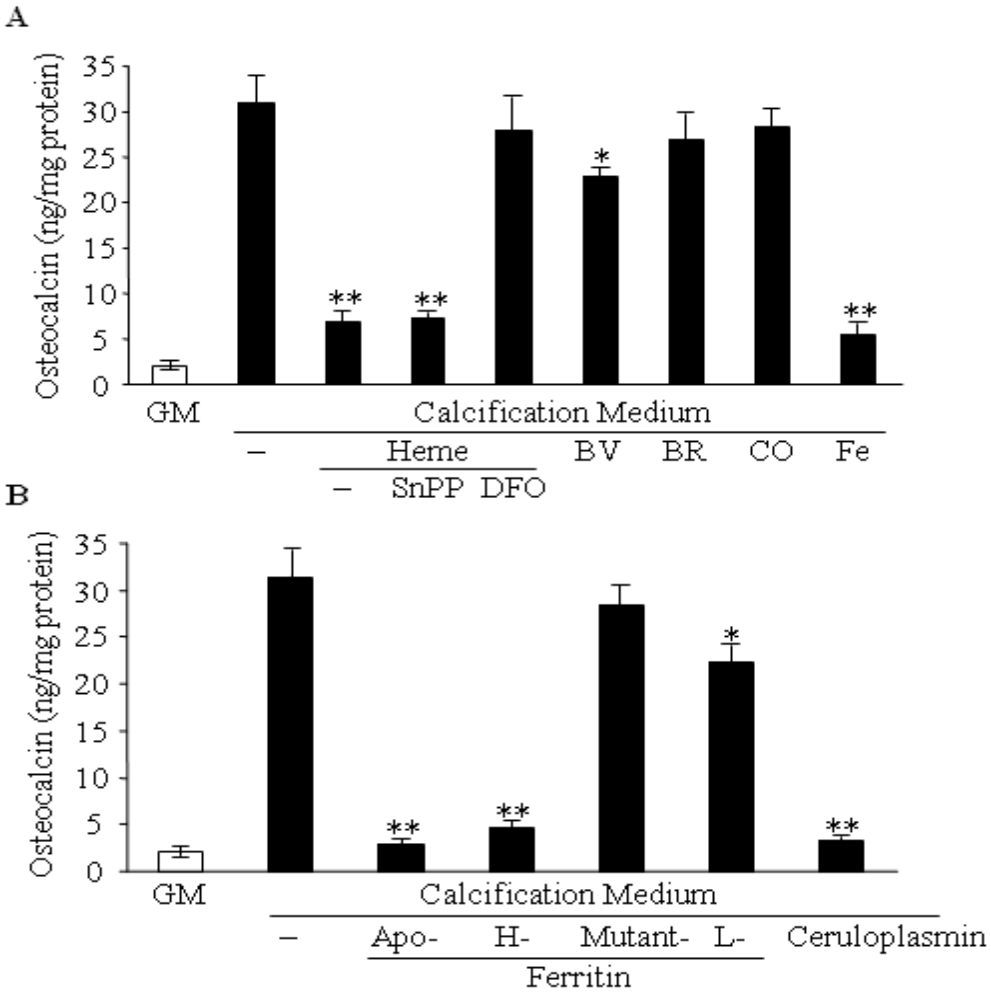


Figure 7. Ferritin attenuates the upregulation of osteocalcin induced by elevated Pi. (A and B) SMCs were treated as described at Figure 6, and osteocalcin levels were determined as described in the Methods section. Data are means ± SD of three independent experiments each performed in duplicate. *$P < 0.05$; **$P < 0.01$.

upregulation of osteocalcin similarly to heme. In addition, biliverdin had a mild but significant effect ($p < 0.05$), while other products of HO reaction – bilirubin and CO – failed to downregulate

high Pi induced osteocalcin expression (Fig 7A). Apoferritin, H ferritin and ceruloplasmin abolished expression of osteocalcin while H mutant 222 had no effect at all (Fig 7B).

Finally, to explore the mechanism underlying the inhibition of mineralization, we examined the level of Cbfa-1 the “master gene” of osteoblast differentiation in our *in vitro* model. Culturing SMC in calcification medium for 48 hours resulted in a 1.8 fold increase in Cbfa-1 mRNA level compared to cells maintained in normal growth medium. Heme inhibited induction of Cbfa-1 mRNA ($p < 0.05$). Accordingly, also apoferritin significantly suppressed this Cbfa-1 induction (Fig 8A).

We also tested the intracellular levels of Pi (Fig 8B) and as our results indicate neither apoferritin, nor ceruloplasmin alter intracellular Pi levels after 24 hours. Iron causes slight but significant decrease in the level of intracellular Pi that may be attributed to its phosphate binding capacity. Notice, such decrease in intracellular Pi levels does not result in significant inhibition of calcification. Although there was some inhibition of calcification, the extent was one third of that observed with heme or iron (data not shown).

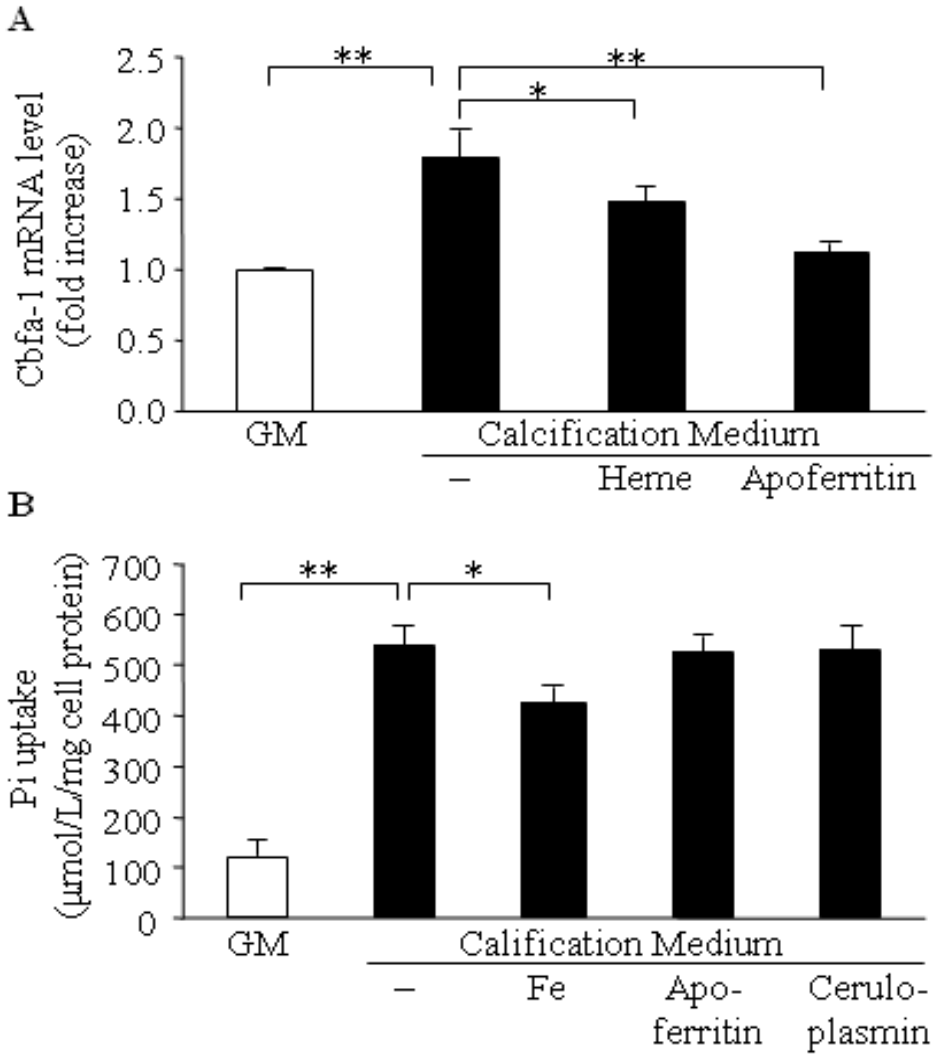


Figure 8. (A) Both heme and apoferritin inhibit elevated Pi-induced increase in Cbfa-1 mRNA level. SMCs were cultured in GM or in calcification medium alone or in the presence of heme (50 µmol/L) or apoferritin (2 mg/ml) for 48 h. Cbfa-1 mRNA levels were determined by quantitative reverse transcription–PCR as described in the Concise Methods section. Data are means ± SEM of five independent experiments performed in triplicate. *$P < 0.05$; **$P < 0.01$. (B) Intracellular Pi concentrations are not affected by apoferritin or ceruloplasmin. SMCs were cultured in GM and calcification medium supplemented by iron (50 µmol/L), apoferritin (2 mg/ml), or ceruloplasmin (4 mg/ml) for 24 h. Cell lysates were used to measure Pi levels. Data are means ± SEM of three independent experiments performed in duplicate. *$P < 0.05$; **$P < 0.01$.

Iron inhibits calcification of OB in a dose responsive manner

To develop an *in vitro* model of osteoblastic activity and mineralization we cultured human OB in calcification medium which was prepared by addition of 2.5 mmol of Pi to the growth medium (GM). Granular deposits developed in OB grown in calcification medium for 14 days (Fig 9A), but not in the control culture grown in normal GM (Fig 9A) as confirmed by von Kossa staining. We found that addition of iron to the calcification medium suppresses granular deposit development (Fig 9A) and extracellular calcium deposition in a dose responsive manner, causing highly significant inhibition at a concentration of 25 µmol/L, and a complete inhibition while using 50 µmol/L of iron (Fig 9B). As iron is a very potent inducer of ferritin (Fig 10A and B), next we tested whether the observed inhibitory effect of iron on calcium deposition is mimicked by apoferritin. We found that iron-free apoferritin at 2 mg/ml dose abolishes granule-formation (Figure 9A) and dose dependently inhibits calcium deposition causing complete inhibition when applied at a concentration of 2 mg/ml (Fig 9C). Then we asked if decreasing the level of available iron with the iron chelator deferoxamine (DFO) – which in turn leads to the posttranscriptional downregulation of ferritin synthesis (Fig 10A and B) –could alter the level of calcification. Indeed addition of DFO (10 µmol/L) to the calcification medium increased the levels of calcium deposition by approximately 20% (Fig 9A, panel v, Fig 9D). In fact, both endogenous up-, and downregulation of ferritin H- and L-chains with Fe or DFO respectively and exogenous administration of apoferritin caused alterations of calcium deposistion of OB. We found a strong negative correlation between the ferritin levels of the cells and the observed calcium depositions (Fig 10C).

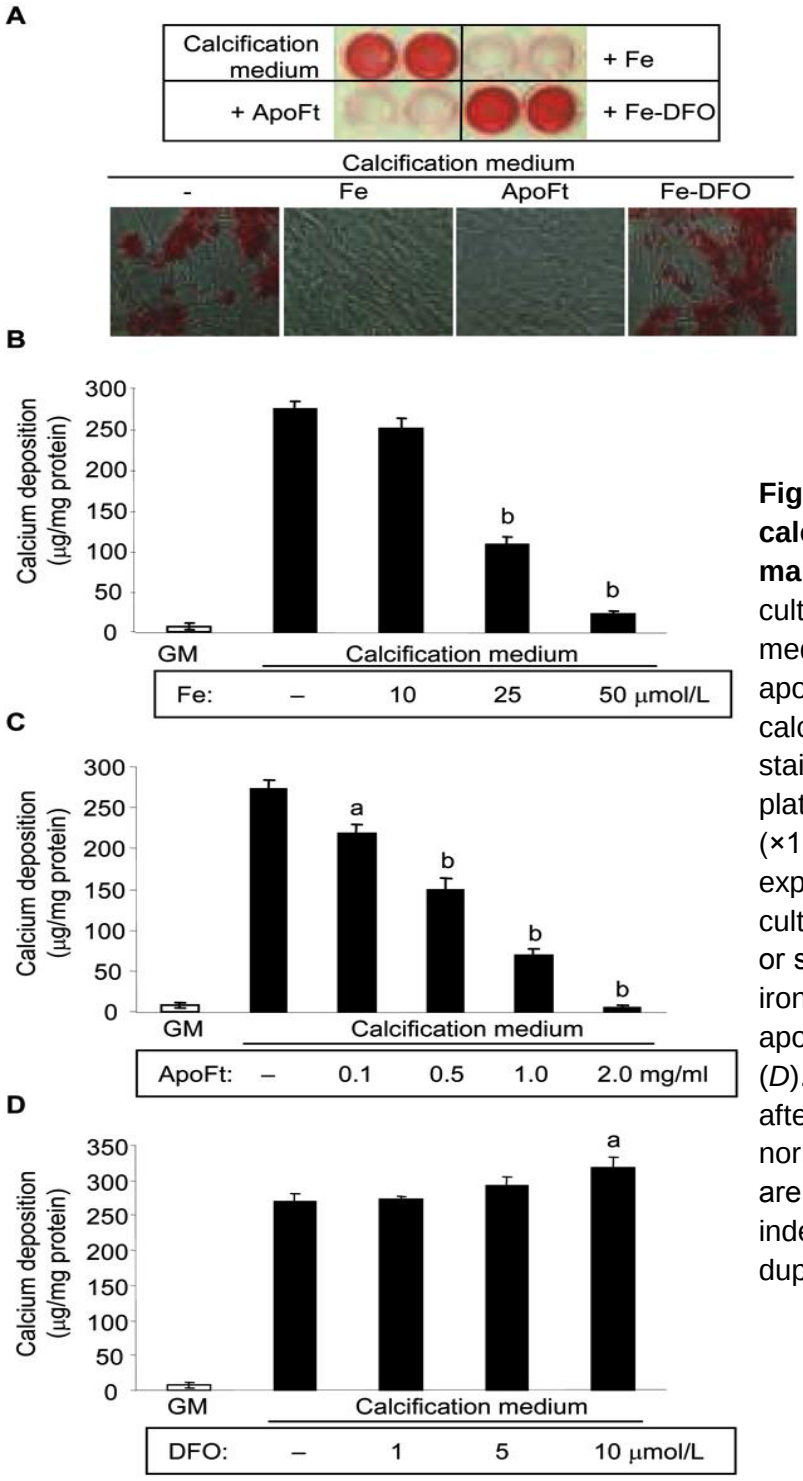


Figure 9. Iron inhibits extracellular calcium deposition in a dose-responsive manner. (A) Human osteoblasts were cultured in growth medium and calcification medium supplemented with iron or apoferritin for 14 days. *In vitro* OB calcification was determined by alizarin red staining. Representative images of stained plates (*upper panel*) and microscopic views (×100, *lower panel*) from three independent experiments are shown. (*B–D*) OBs were cultured in GM or calcification medium alone or supplemented with 10, 25, or 50 µmol/L or iron (*B*); 0.1, 0.5, 1, and 2 mg/mL of apoferritin (*C*); or 1, 5, and 10 µmol/L of DFO (*D*). Calcium content of cells was measured after 14 days' incubation, and the level was normalized by protein content of cells. Data are presented as mean ± SD of three independent experiments performed in duplicates. [a]$p < .05$. [b]$p < .01$.

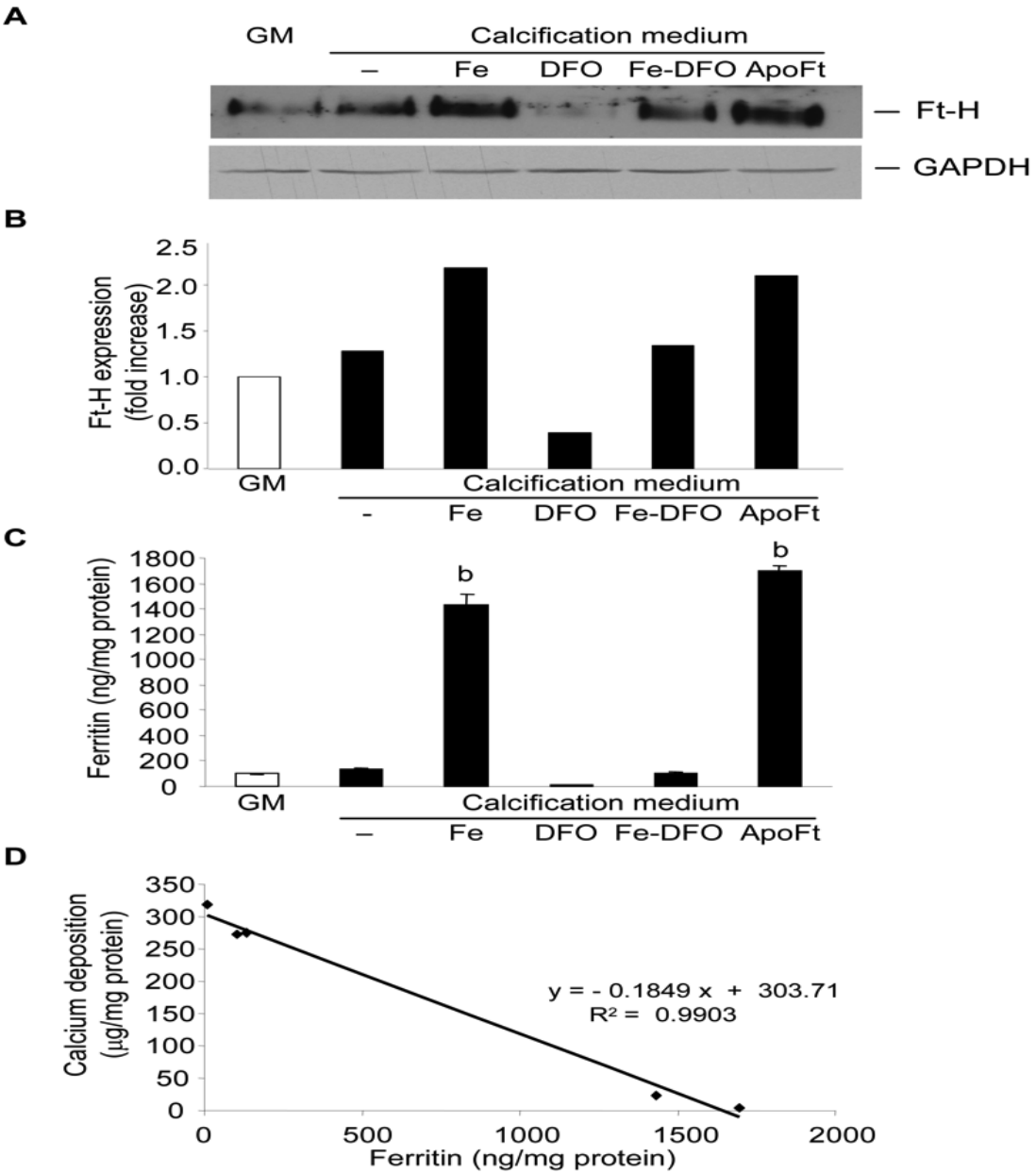


Figure 10. Iron inhibits OB mineralization via induction of ferritin. OBs were cultured in GM or calcification medium alone or in the presence of iron (50 µmol/L), DFO (10 µmol/L), iron + DFO (50 µmol/L each), and apoferritin (2 mg/mL) for 14 days. (*A*) Representative Western blot shows expression of ferritin H (Ft-H). Same cell lysates were electrophoresed in 12.5% SDS-PAGE to detect GAPDH and show equal loading of protein. (*B*) Densitometric measurement of the band intensities for ferritin H was normalized to GAPDH and is representative of three independent experiments. (*C*) Cell lysate was used to quantify ferritin expression by an immunoassay. Graph shows mean ± SD of three separate experiments performed in duplicate. (*D*) Strong negative correlation between ferritin levels of cells and calcium deposition in OBs cultured in calcification medium alone (♦) or supplemented with iron (▲), DFO (●), iron + DFO (▪), or apoferritin (▼). $^{b}p < .01$.

Ferroxidase activity is responsible for inhibition of calcification

To investigate whether the inhibitory effect of ferritin is due to its iron sequestration capacity or its ferroxidase activity or both we tested the effect of ceruloplasmin, a protein, which possesses ferroxidase activity but not iron sequestration capacity. In fact as indicated in Fig.11A exogenous ceruloplasmin dose dependently inhibited mineralization causing abolishment of

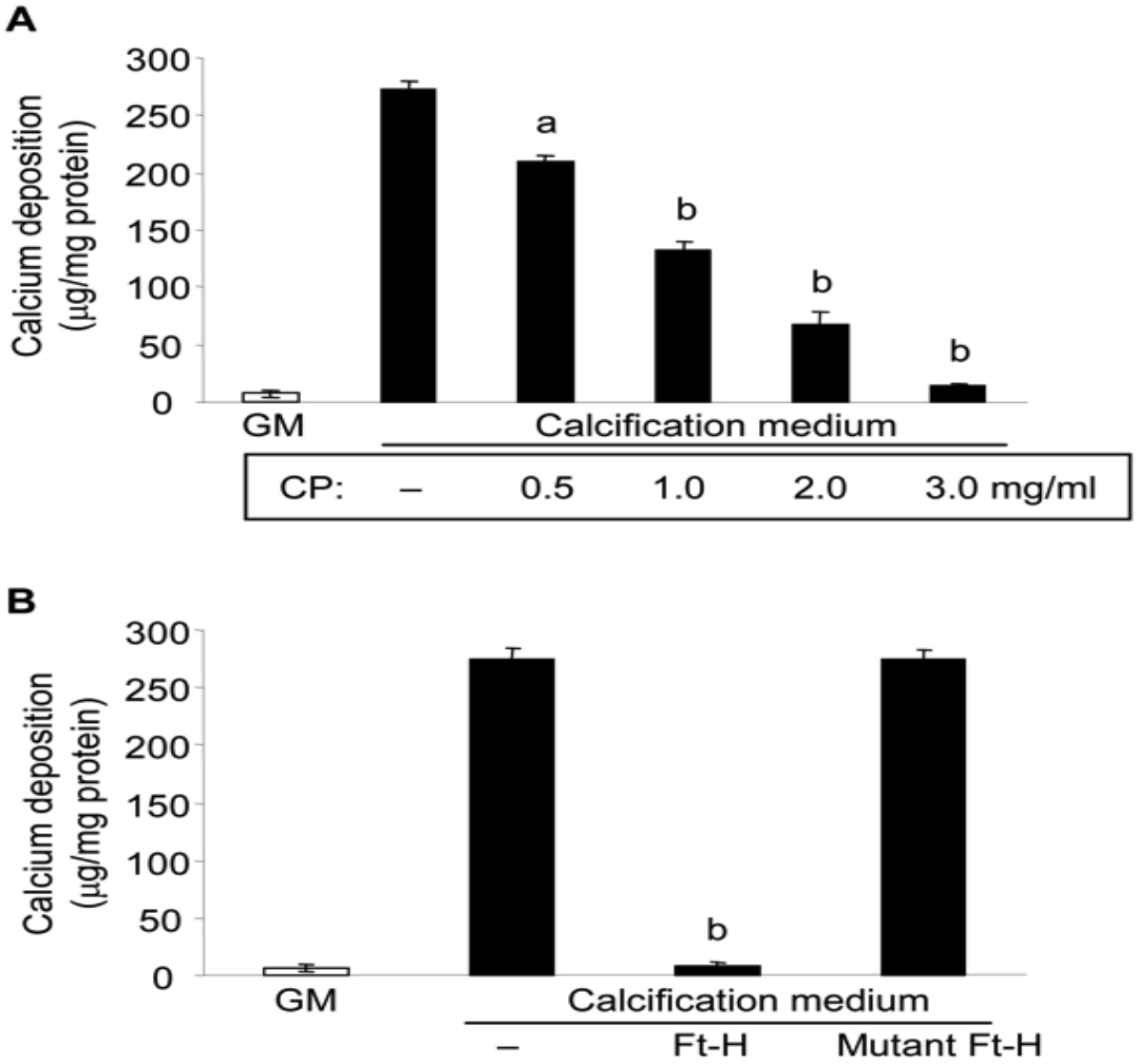


Figure 11. Ferritin ferroxidase activity is responsible for inhibition of calcification. OBs were cultured in GM or calcification medium for 14 days, and the calcification medium were supplemented with 0.5, 1, 2, and 3 mg/mL ceruloplasmin (CP) (A) and H-ferritin or mutant 222 ferritin (Mutant Ft-H) 2 mg/mL. (*B*) Calcium deposition was measured after 14 days and normalized by cellular protein. Data are presented as mean ± SD of five independent experiments performed in duplicates. [a]$p < 0.05$. [b]$p < .01$.

calcium deposition at a dose of 3 mg/ml. Experiments using recombinant H-ferritin and the H-mutant 222 ferritin which lacks both ferroxidase activity and iron storing capability provided further evidence of the role of ferroxidase activity in the observed inhibitory effect, while H-ferritin attenuated calcium deposition at a dose of 2 mg/ml, the H-mutant 222 ferritin did not alter calcification at all when applied at the same dose (Fig 11B).

Ferritin-ferroxidase activity downregulates expression and subsequent activity of ALP

To investigate whether the observed effects are only restricted to reduced calcification or they influence OB gene expression as well, we examined the role that ferritin may play regarding OB specific genes. First we examined the ALP gene since its activity is a good marker of osteoblastic activity. ALP activity is crucial in early osteogenesis by increasing local levels of Pi and therefore facilitating the formation of hydroxyapatite crystals.

Basal ALP activity of OB was quite high when culturing them in a normal GM and addition of Pi to the GM caused a mild but significant increase in ALP activity (Fig 12A). However addition of iron to the high Pi containing calcification medium led to a significant decrease of ALP activity, which became much lower than the basal ALP activity of OB cultured in GM. Accordingly, this inhibitory effect was attenuated when using equimolar concentrations of iron and DFO together. Additon of 10 μmol/L of DFO alone caused mild but significant increase in the level of ALP activity (Fig 12A). Supplementation of the calcification media with apoferritin caused a dose responsive inhibition of ALP expression and activity (Fig 12B and C). The role of ferroxidase activity behind the observed inhibitory effect of iron and ferritin was also assessed by using wild type and mutant H-ferritin and ceruloplasmin. While addition of both H-ferritin and ceruloplasmin to the calcification medium strongly downregulated ALP activity, H- mutant 222 ferritin did not influence ALP activity at all (Fig 12D) confirming ferroxidase activity as the central element of this inhibition.

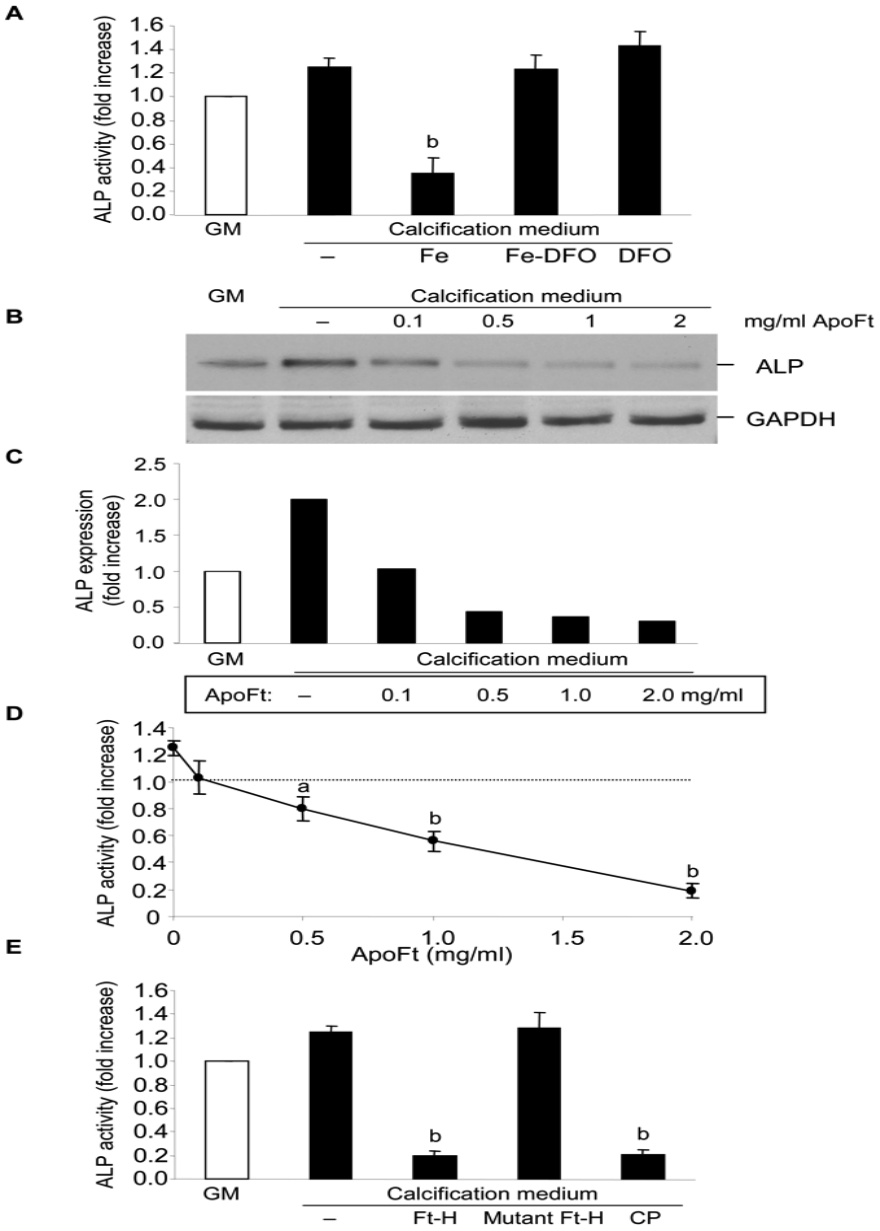


Figure 12. Suppression of ALP expression and activity by ferritin/ferroxidase activity. (A) OBs were cultured in GM or calcification medium alone or in the presence of iron (50 µmol/L), DFO (10 µmol/L), or iron + DFO (50 µmol/L each) and apoferritin (2 mg/mL) for 14 days. ALP activity of cells was measured as described in Methods. Data are expressed as means ± SD of five independent experiments each performed in duplicates. $^{b}p < .01$. $^{a}p < .05$. (*B*) OBs were cultured in GM or calcification medium alone or supplemented with 0.1, 0.5, 1, and 2 mg/mL of apoferritin for 14 days. Representative of three Western blots shows ALP expression. Membrane was reprobed for GAPDH to show equal loading of proteins. (*C*) Densitometric measurement of the band intensities for ALP was normalized to GAPDH and presents measurements from three independent experiments. (*D*) OBs were cultured as in (*B*), and ALP activity was measured. Data show average ± SD of three independent assays performed in triplicate. (*E*) OBs were cultured in GM or calcification medium alone or supplemented with recombinant ferritin H-chain wild-type or mutant 222 (2 mg/mL) or ceruloplasmin (3 mg/mL) for 14 days. ALP activity was measured and is shown as mean ± SD of three independent experiments done in duplicates.

Ferritin-ferroxidase activity suppresses the exclusive OB product: Osteocalcin

While maintaining OB in calcification medium for 14 days results in ~ 13 fold increase in the amount of osteocalcin deposition in the newly synthesized extracellular matrix, addition of iron decreased the level of osteocalcin dose responsively and causing very significant inhibition at doses of 25 µmol/L and almost a complete inhibition at a dose of 50 µmol/L (Fig 13A).

Accordingly, supplementation with apoferritin also caused a dose dependent inhibition in the amount of osteocalcin providing very significant inhibition at 0.5 mg/ml and a complete inhibition was resulted at a dose of 2 mg/ml (Fig 13B). To further confirm that this inhibition is mainly provided by ferroxidase activity, ceruloplasmin and H-ferritin (wild type and mutant 222) were added to the calcification medium. While ceruloplasmin and H-ferritin abolished osteocalcin production, H-mutant 222 ferritin did not cause any significant inhibition regarding the amount of osteocalcin deposition (Fig 13C).

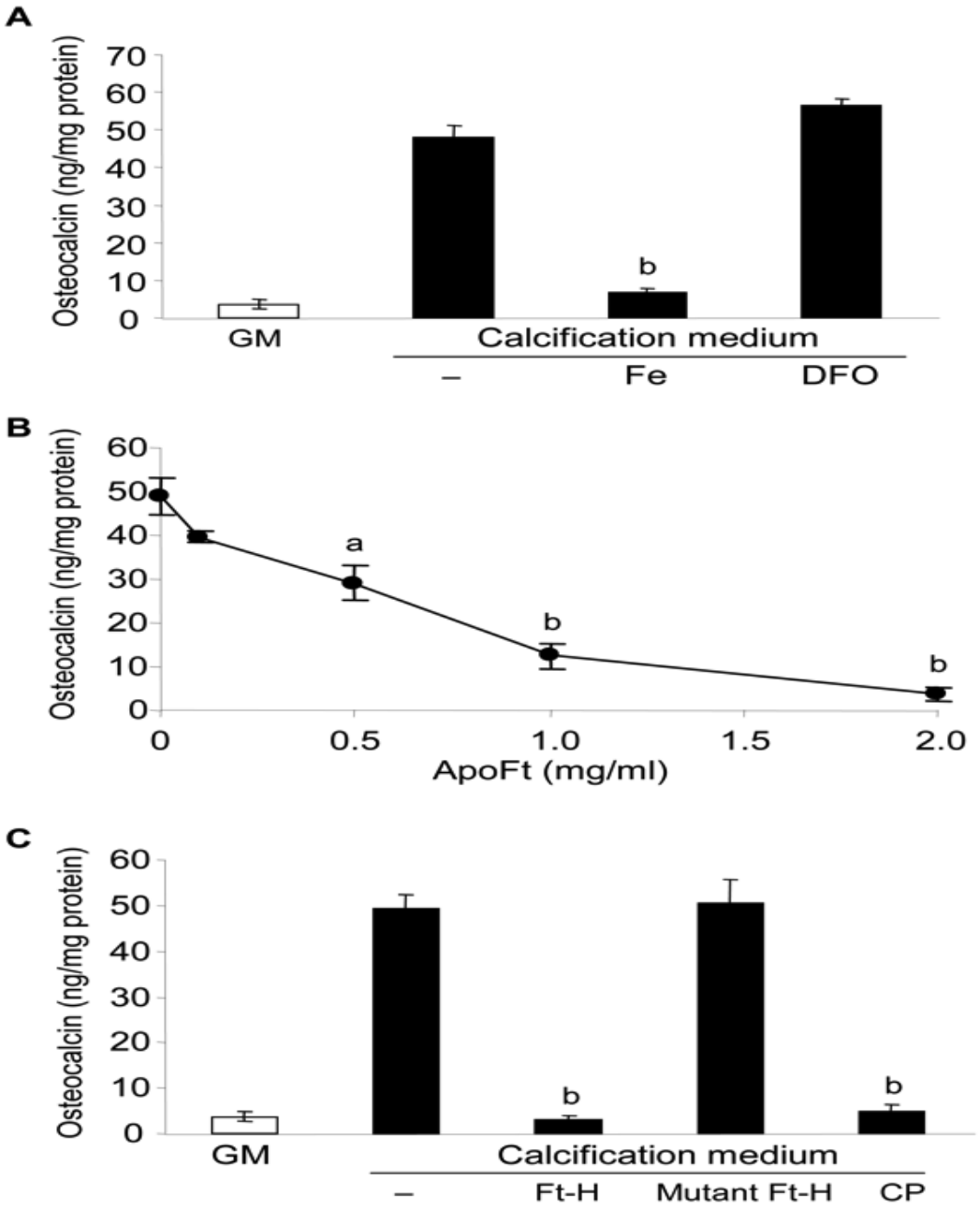


Figure 13. Ferritin attenuates upregulation of osteocalcin. OBs were cultured in GM or calcification medium alone or supplemented with iron (50 µmol/L) or DFO (10 µmol/L) (*A*), apoferritin (0.1, 0.5, 1, or 2 mg/mL) (*B*), and recombinant ferritin H-chain or mutant 222 at a dose of 2 mg/mL or ceruloplasmin 3 mg/mL (*C*) for 14 days. The extracellular matrix was dissolved, and osteocalcin deposition was quantified as described in methods. Data derived from three separate experiments performed in triplicates and shown as mean ± SD. $^{b}p < 0.01$. $^{a}p < 0.05$.

Intracellular Pi concentrations are not affected by apoferritin or ceruloplasmin

Because ferric iron is known to bind phosphate and to examine the mechanism by which both mineralization and OB gene expression are downregulated, we measured Pi uptake of OBs after 24 hours of incubation in calcification medium. Our results indicate (Fig. 14A) that iron caused a mild but significant decrease in the level of intracellular Pi. This is probably due to the phosphate-binding capacity of ferric iron. On the contrary, neither apoferritin nor ceruloplasmin altered intracellular Pi concentrations 24 hours after incubation. Hence iron is capable of binding to phosphate and hence causing a minor decrease in the level of extracellular Pi, but this seems to contribute only in minor proportion to the mechanism of action of iron on bone deposition. This notion is firmly confirmed by the inhibition caused by apoferritin, H-ferritin, and ceruloplasmin, which are all free of iron.

Ferritin inhibits OB specific transcription factor Cbfa-1

It is well recognized that OB maturation and differentiation is powerfully influenced (if not dictated) by transcription factor Cbfa-1. Hence we investigated if ferritin could downregulate expression of Cbfa-1 and therefore put forward a possible explanation for the observed inhibitory effects of ferritin on OB activity. Indeed, supplementation of the calcification medium with apoferritin caused a dose dependent downregulation of Cbfa-1 protein expression (Fig 14).

Ferritin prevents calcification of human 143-B cells

In order to confirm the inhibitory effect of ferritin and ferroxidase activity on mineralization, we employed another human osteoblastic cell line: 143-B osteosarcoma cells. Granular deposits developed in 143-B cells grown in calcification medium for 7 days (Fig. 15A) but not in the control culture grown in normal GM, as demonstrated by alizarin red staining. We found that addition of iron to the calcification medium prevented granular deposit development and extracellular calcium deposition at a concentration of 50 µmol/L (see Fig. 15A, B). Furthermore,

exposure of cells to apoferritin also abolishes granule formation and completely inhibits calcium deposition when applied at a concentration of 2 mg/mL (see Fig. 15A, B). Importantly, cells treated with ceruloplasmin fail to exhibit granule formation and extracellular calcium accumulation at a concentration of 3 mg/mL. These results confirm that ferroxidase activity acts as an inhibitor in OB mineralization.

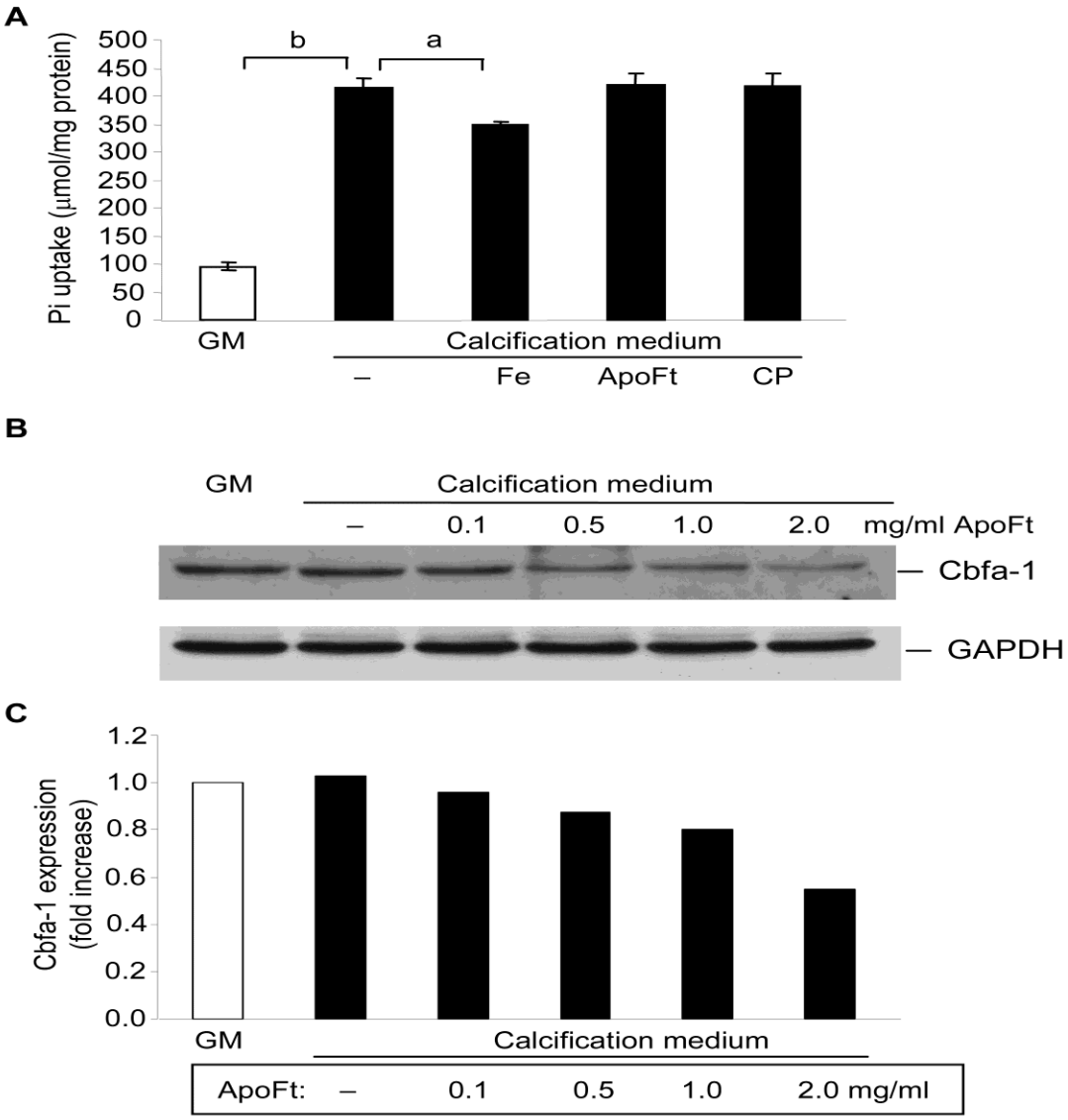


Figure 6

Figure 14. Intracellular Pi concentrations are not affected by apoferritin or ceruloplasmin. (A) OBs were cultured in GM or in calcification medium alone or supplemented with iron (50µmol/L), apoferritin (2 mg/mL), or ceruloplasmin (3 mg/mL) for 24 hours. Cell lysates were used to measure Pi levels, as described in the methods. Results are presented as mean_SD of three independent experiments performed in duplicates. Cbfa-1 levels are suppressed in a dose-responsive manner by apoferritin supplementation. (B) After 14 days culturing of OBs in GM or calcification medium

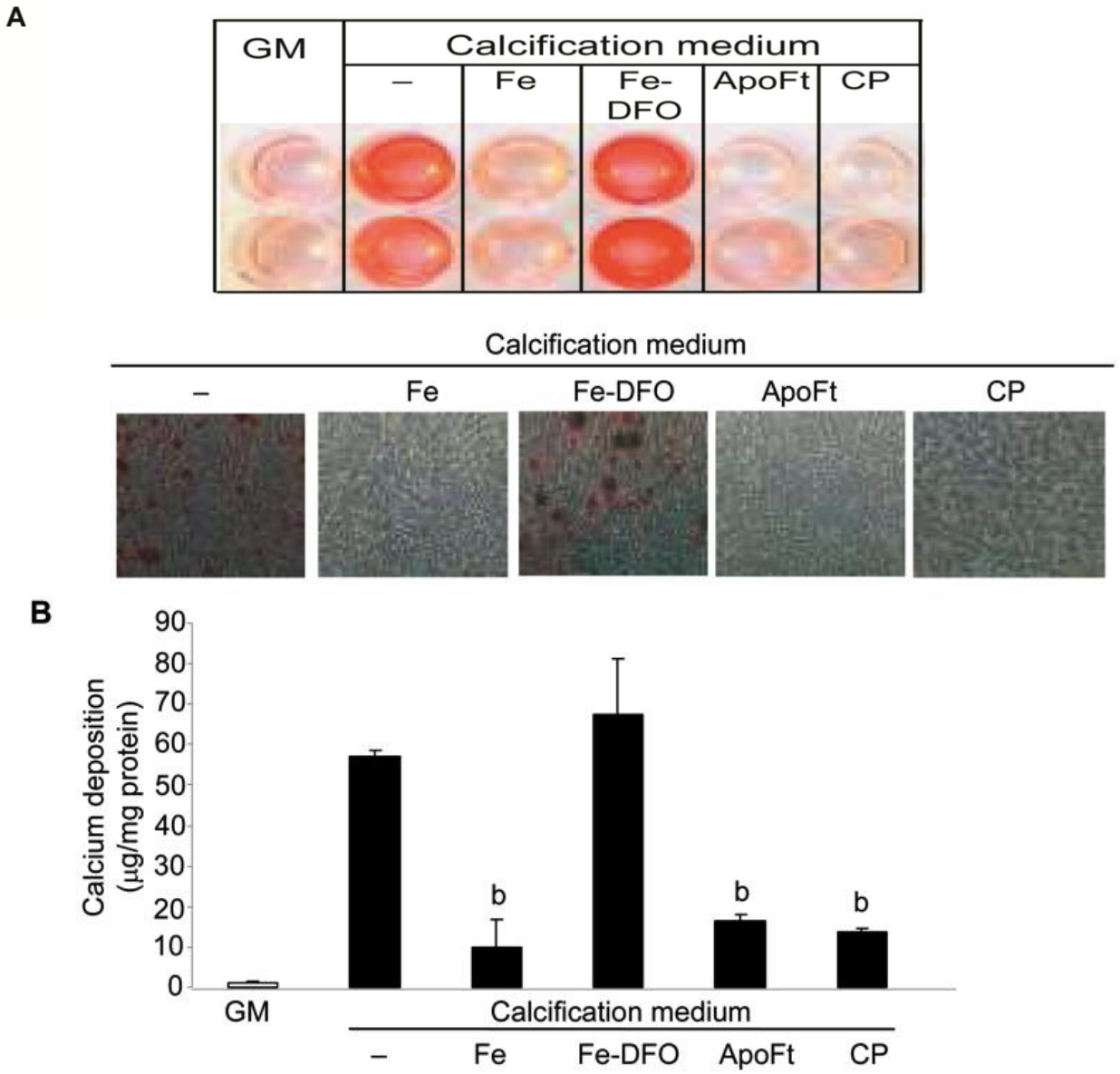


Figure 15. Ferritin prevents calcification of human 143-B cells. (*A*) 143-B cells were culture in growth medium and calcification medium alone or supplemented with iron (50 µmol/L), iron with DFO (both are 50 µmol/L), apoferritin (2 mg/mL), or ceruloplasmin (3 mg/mL). Granular deposits were determined by alizarin red staining. Representative images of stained plates (*upper panel*) and microscopic views (×100, *lower panel*) from three independent experiments are shown. (*B*) After 7 days of culturing of 143-B cells, extracellular calcium deposition was determined and was normalized to protein level. Data are presented as mean ± SD of three independent experiments performed in duplicates. $^{b}p < .01$.

DISCUSSION

Part 1

The studies that are presented in this thesis have focused on two different, yet overlapping clinical conditions. First, our findings have focused to provide molecular understanding into the mechanism of inhibition of high Pi induced vascular calcification. Our results suggest that induction of ferritin is a potent inhibitor of SMC calcification and transdifferentiation into osteoblast like cells. Second, we sought to investigate the mechanism of iron overload induced osteoporosis. We provide evidence that iron inhibits osteoblast activity via upregulation of intracellular ferritin. In both studies we found that ferroxidase activity plays a central role in inhibition of vascular calcification and osteoblast activity. These studies, for the first time, provide mechanistic insight into two very detrimental clinical conditions and hence offer novel avenues of understanding and future research that may lead to innovative therapeutic modalities.

The most common cause of death in patients with ESRD is related to cardiovascular disorders (64). In this regard vascular calcification, particularly extensive coronary artery calcification, is being increasingly recognized as a major culprit of high mortality in this group of patients (19, 65). Overall, vascular calcification follows two distinct patterns. (i) Intimal calcification that occurs with atherosclerotic plaques and (ii) medial calcification, which is characterized by diffuse calcification of the media, particularly at the level of the internal elastic lamina, that does not necessarily accompany atherosclerosis. The latter is commonly observed in patients with ESRD. In fact, the presence of medial calcification in younger patients requiring renal replacement therapy has been established (18, 19). Vascular calcification was traditionally considered a passive process that was merely an outcome of calcium-phosphate product formation and deposition, a process that was enhanced in an alkaline environment. This notion has recently been strongly challenged and there is now strong evidence that identifies the

process of vascular calcification very similar to ossification (66, 67). Such process requires transdifferentiation of the medial layer SMC into cells that express similar genes to osteoblasts providing a favorable microenviroment for deposition of hydroxyapatite crystals and calcification of vascular tree. Osteogenic genes such as alkaline phosphatase, osteocalcin and collagen-I have all been identifies in SMC that have undergone this transition (32, 66, 67). Expression of the above genes is regulated by a transcription factor core binding factor alpha-1 (Cbfa-1) that has also been demonstrated to be upregulated during the transition of SMC into osteoblast like cells (68). The significance and fundamental role of this transcription factor has been highlighted in transgenic animals that do not harbor the Cbfa-1 gene (69). This knock-out approach results in disruption of both endochondral and intramembranous ossificiation. In addition to the pathomechanism of vascular calcification, extensive research has also identified a number of culprits that stimulate such process as well as defense mechanisms that the SMC harbor to prevent osteoblastic transdifferentiation (28). Decline in renal function is accompanied by a number of derangements including accumulation of Pi that is distinctively recognized as a major inducer of vascular calcification in ESRD patients (32, 34, 35, 70). In fact, analysing data from hemodialysis patients reveals that the extent of elevated serum phosphate is positively correlated with mortality (33, 71, 72). Elevated level of Pi has been shown to stimulate upregulation of a sodium dependent co-tarnspoter, Pit-1 (73). The activity of Pit-1 leads to increment of intracellular Pi concentration and initiation of a cascade of gene expression, ultimately leading to medial calcification. Based on numerous in-vitro, animal and clinical studies it is now evident that hyperphosphatemia is a major risk factor for developing vascular calcification in patients with ESRD (70, 74). The complications of increased calcification have been documented and there is mounting evidence that it is highly associated with increased mortality and morbidity.

Despite extensive advances in our understanding of mechanisms leading to vascular calcification, preventive and/or therapeutic agents that would inhibit vascular calcification are yet

to be identified and verified. To this end, we were prompted to examine the potential role of HO-1/ferritin system in vascular calcification. Upon its discovery, HO-1 was introduced as an enzyme whose function was solely to convert heme into bilirubin (36). It is now known that in biological systems oxidation of heme is carried out by two isozymes, HO-1 and HO-2. HO-1 is the highly inducible form and HO-2 appears to be uninducible (37). Furthermore, based on extensive evidence we have recognized that during the conversion of heme to bilirubin other byproducts, namely, carbon monoxide and biliverdin are formed. Additionally, oxidation of heme releases the iron at the core of the porphyrin ring which in turn strongly induces the expression of ferritin. In addition to the discovery of other isozymes and byproducts of the pathway, a number of pivotal studies in the early 1990s changed our understanding of this enzyme system. Balla and colleagues provided the first line of evidence that revealed the potential toxicity of heme and its ability to oxidize LDL which is a major initial pathologic step in atherosclerosis (75). A subsequent study showed that HO-1 is rapidly and robustly following a rhabdomyolysis induced acute kidney injury model (76). The above induction coupled with synthesis of ferritin provided a strong anti-oxidant response (76). More evidence followed after Ball et al. demonstrated that induction of ferritin plays a major role in protecting endothelial cells against oxidative injury (52). This set of original work led to a considerable wave of interest and investigation in the field of HO-1/ferritin system. In accordance to a strong body of evidence, this enzyme system is now recognized an essential endogenous protective machinery with beneficial effects that have been documented in numerous clinical conditions such as sepsis, acute kidney injury, ischemia-reperfusion injury among others (77). While immediate removal of heme, distinctly recognized for its pro-oxidant nature, is a significant step in minimizing injury, other protective properties of the HO-1/ferritin system have been attributed to one or more byproducts of heme degradation namely, biliverdin, carbon monoxide, bilirubin and ferritin (41, 42, 77). Within the context of cardiovascular system evidence indicates that upregulation of HO-1 and ferritin occurs in the early phase of progression of atherosclerotic lesions, (78, 79)

possibly reflecting cellular response to heme and/or heme-iron–generated lipid peroxidation products. There is growing evidence that induction of the HO-1/ferritin system is protective against atherosclerosis (80). Upregulation of HO-1 and ferritin inhibits cytotoxicity induced by oxidized LDL in endothelial cells (81) and atherosclerotic lesion formation in LDL receptor knockout mice, whereas inhibition of HO enzyme activity by SnPP leads to accelerated atherosclerosis in these mice (82). The extent of evidence in this system encouraged us to examine whether the induction of HO-1/ferritin system may play a beneficial role in vascular calcification.

First, we confirmed that growing SMC in Pi-containing calcification medium causes mineralization in a time-dependent manner, and in agreement with other findings, we observed marked induction of specific osteoblast cell markers such as ALP and osteocalcin during the culture period, supporting the notion that such calcification is an active cell mediated process. Using this *in vitro* model we found that addition of heme had an inhibitory effect on SMC mineralization in a dose dependent manner. This observation incited us to hypothesize that one or more products of heme degradation mediate the process of SMC mineralization. To test this hypothesis and to identify the mediator for inhibition of SMC calcification and osteoblastic transformation, we first examined the individual products of heme degradation by HO. Iron almost completely attenuated extracellular calcium deposition as well as upregulation of osteocalcin and ALP. Biliverdin was less effective, whereas bilirubin and CO failed to alter mineralization. Then we analyzed the possible role of ferritin that is also strongly upregulated by heme. We examined whether exogenous ferritin affected mineralization, in accordance with the report that demonstrated the uptake of exogenous apoferritin in a dosage-responsive manner (52). We found that apoferritin caused a suppression of SMC mineralization in a dose responsive manner. Furthermore, exogenous H-ferritin and ceruloplasmin—two largely different proteins that share only ferroxidase activity—showed the same suppression of osteoblastic differentiation. The importance of ferroxidase activity in the process was also verified by the

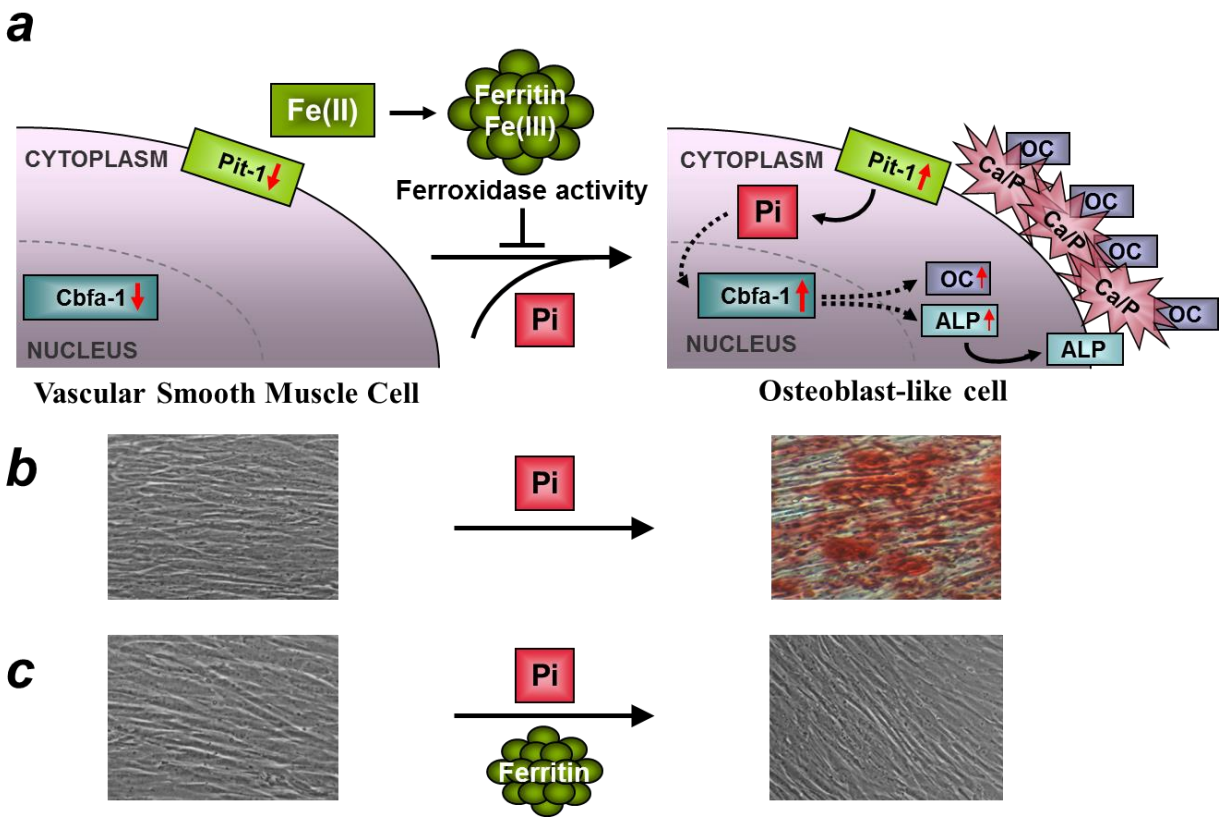
finding that a structurally analogous molecule to H-ferritin, namely the recombinant H-ferritin mutant 222, which lacks ferroxidase activity and iron storage capability, was ineffectual. These results strongly support the notion that inhibition of mineralization may be attributed to ferritin and its ferroxidase activity.

Upregulation of H- and L-chains of ferritin in cells exposed to heme is driven at the translational level via labile iron provided from heme catalysis by HO (83). In addition, heme itself enhances ferritin expression by increasing its translational rate (84). This explains why induction of ferritin is not affected by inhibition of HO activity in cells exposed to heme, as observed in previous studies (52). Accordingly, in this study, cells treated with heme in the presence of SnPP or siRNA for HO-1 exhibited very low HO activity but high ferritin level. Treatment of cells with SnPP or siRNA for HO-1 did not affect heme-mediated ferritin induction and did not influence heme-mediated inhibition of mineralization. These results indicate that ferritin alone is capable of preventing SMC calcification and differentiation after cells are exposed to heme irrespective of the activity of the HO enzymes. To confirm further the role of ferritin in the heme-induced inhibition of SMC mineralization, we selectively downregulated heme-induced ferritin synthesis by the iron chelator DFO, which led to substantial loss of inhibition of calcification.

A great deal has been investigated and documented about the mechanism(s) of vascular calcification and increased expression of Cbfa-1 is implicated in the transition of SMCs into osteoblast-like cells (35, 85). The observation that heme or apoferritin significantly suppressed Cbfa-1 induction by high Pi indicates that inhibition of mineralization by ferritin might occur via transcription factor Cbfa-1. While traditionally regarded as a cytoplasmic protein, H-ferritin has been shown to localize to the nucleus whereby not only it sequesters iron leading to protective effects against oxidative damage to the DNA, it is also involved in the regulation of transcription of certain genes such as beta globin (86-89). Therefore, whether ferritin alters the expression of the “master” of osteoblast transformation, Cbfa-1 was examined. Results indicated that while Pi

upregulates the expression of Cbfa-1 in SMC, ferritin attenuates such upregulation. A relationship between calcification and iron metabolism has never been explored, although it should be noted that most patients who have CKD and require renal replacement therapy have vascular calcification (33, 71, 72) and deranged iron homeostasis (90). Moreover, in patients with CKD, there is an accumulation of iron in reticuloendothelial cells that is accompanied by higher levels of plasma ferritin; however, this increase largely results because most of such iron is sequestered by reticuloendothelial cells and its availability to other cells is significantly reduced. This translates to depletion of intracellular ferritin and subsequent anemia of chronic disease. In inflammatory diseases such as CKD, cytokines released by activated leukocytes and other cells exert multiple effects (91). These contribute to the reduction in hemoglobin levels and increased hepatic synthesis of hepcidin that in turn binds to ferroportin, the transporter that allows egress of iron from reticuloendothelial macrophages and from intestinal epithelial cells (92, 93). Binding of hepcidin leads to internalization and degradation of ferroportin. The corresponding sequestration of iron within the macrophages limits iron availability to all cells (92, 93). On the basis of our observations, we suggest that such derangements in iron metabolism may facilitate Pi-induced vascular calcification; therefore, parenteral iron administration may be considered not only to replete iron and correct anemia but also to prevent vascular calcification via increasing intracellular ferritin expression and decreasing extracellular Pi level, especially when the inflammation is well controlled.

In conclusion, findings presented highlight for the first time a novel role for ferritin in the context of SMC mineralization. These results provide new insights into the mechanisms of vascular calcification and uncover the HO-1/ferritin pathway as a target for new strategies to prevent vascular calcification.



Scheme 5. The inhibitory effect of ferritin on SMC osteoblastic transdifferentiation..

(a) Scheme of Pi-mediated osteoblastic transformation of SMC. Red arrows represent response to elevated Pi. Elevated Pi increase the expression of Pit-1 resulting in increased uptake and elevated cytosolic Pi concentration followed by upregulation of the bone specific transcription factor Cbfa-1. Subsequently, expressions of Cbfa-1-regulated target genes, e.g. osteocalcin (OC) and alkaline phosphatase (ALP) are increased. ALP and is then secreted in matrix vesicles and initiate mineralization. Extracellular matrix buildup of Ca/P and OC. Ferritin via its ferroxidase activity inhibits the main steps of SMC transdifferentiation into osteoblast type cells. Pi-induced phosphate uptake, upregulation of Pit-1, Cbfa1, ALP and OC expression as well as Ca deposition are all inhibited by ferritin.

(b) Pi-mediated mineralization of SMC calcium deposition assessed by alizarin red staining of SMC cultured under normal or elevated Pi conditions.

(c) Effect of ferritin on Pi-mediated mineralization of SMC. Representative images of alizarin red staining of SMC (100x) exhibiting elevated ferritin expression cultured under normal or elevated Pi conditions.

Part 2

Following the study discussed above we asked whether the mechanism of ferritin induced inhibition of mineralization may be responsible for the decreased bone mass that is commonly documented in patients with iron overload.

In this study following we examined the process of osteogenesis *in vitro* by growing human OBs in Pi-rich medium, and in particular, we studied the effect of iron. Our findings strongly suggest that the inhibition of OB activity that occurs with iron supplementation is mainly due to the iron-mediated induction of H-ferritin and its ferroxidase activity. By using DFO, we demonstrated that the iron sequestration capability of ferritin is not responsible for the observed effects. The inhibition occurs after the addition of recombinant H-ferritin and ceruloplasmin, proteins with ferroxidase activity, but not by H-ferritin mutant with inactivated ferroxidase activity. Here we show that ferritin/ferroxidase activity plays a crucial role in downregulation of mineralization and the underlying gene expression.

Bone is a dynamic, highly vascularized tissue with a unique internal repair capacity to heal and remodel without scarring. Several studies have suggested a link between excessive and misplaced iron and secondary decreased bone mass (61, 94-96). In a study of iliac crest biopsies from 21 individuals with severe osteoporosis (at least one vertebral fracture), iron bone concentration (cortical and trabecular) was evaluated using inductively coupled plasma optical emission spectrometry. A significant increase in iron content in cortical bone was found in osteoporotic patients versus 12 controls (97). There is a growing body of evidence that iron can play a deleterious role in bone. In genetic hemochromatosis, previous human studies have found Perl's Prussian blue staining (which unambiguously identifies iron) in bone trabeculae of patients (59, 98). Moreover, bone mineral density is decreased in patients and animal models with genetic hemochromatosis and severe iron overload (58, 96, 99) and negatively correlates

with hepatic iron concentration (a good index of total-body iron overload) and bone mineral density at the femoral neck (60).

A study of iron overload with intramuscular iron dextran was conducted in pigs over 36 days (100). The main effect was a decrease in bone formation without significant changes in bone resorption. Similarly, osteopenia was induced in Sprague-Dawley rats fed a diet containing iron lactate (5%) for 2 or 4 weeks (101). Other lines of evidence firmly confirm the close relationship between iron metabolism and osteogenesis. For instance, lactoferrin, an iron-binding glycoprotein present in epithelial secretions such as milk and in the secondary granules of neutrophils, is suggested as a potent regulator of bone cell activity, and it was reported to increases bone formation *in vivo* (102, 103). Additionally, there is evidence that a green tea iron chelator, epigallocatechin-3-gallate, stimulates mineralization of murine bone marrow mesenchymal stem cells (104). Clinical reports also show that patients with hemolytic anemias, including thalassemia and sickle-cell anemia, eventually develop iron overload. There is strong relationship between such elevated iron levels and the occurrence of osteopenia and osteoporosis in these patients (105-108).

DFO is a powerful chelator of iron, zinc, cobalt, and copper, and it is used commonly to prevent iron overload. Although this may be considered to increase osteogenesis by decreasing iron and subsequent ferritin downregulation, some studies suggest that in addition to the growth retardation owing to untreated thalassemia, DFO produces a further negative effect on growth velocity by causing bone dysplasia. The decrease in growth velocity mainly affects the long bones, in particular, the distal femoral physis, which normally accounts for 70% of femoral growth. It should be noted that these trace metals are more likely to be chelated in the presence of reduced iron levels. Actually, serum zinc levels were below normal limits in 37% of chelated patients in one study (109). Zinc deficiency is associated with delayed skeletal maturation and a reduction in growth, as well as bone matrix and collagen synthesis. In fact, iron chelation with DFO and simultaneous zinc supplementation have been shown to increase growth in some

thalassaemic patients. This further verifies that decreased iron levels can have a beneficial effect on OB activity.

Our study has focused on shedding new light on better understanding the mechanism by which iron causes repression of OB activity and indicates that such derangement is largely due to ferritin and its ferroxidase activity. In agreement with previous studies, we confirm that iron inhibits OB activity in a dose-responsive manner *in vitro*. However, our investigations clearly indicate that such inhibition is mainly due to iron-induced upregulation of ferritin and ferroxidase activity. This notion is supported by the fact that apoferritin, which contains very little iron, if any, causes a dose-responsive decrease of OB gene expression and subsequent calcification. As mentioned above, we have demonstrated dose-responsive uptake of apoferritin previously (52), and this study confirms it. Inhibitory effects of apoferritin can be mimicked with ceruloplasmin. This is an enzyme synthesized in the liver containing six atoms of copper in its structure that carries approximately 90% of the copper in plasma. However, we used this protein in our study because of its well-known ferroxidase activity. The importance of ferroxidase activity in the process was further confirmed by the finding that while recombinant H-ferritin had the same effects observed with apoferritin and ceruloplasmin, a structurally analogous molecule to H-ferritin, namely, the recombinant H-ferritin mutant 222, which lacks ferroxidase activity and iron-storage capability, was completely ineffectual in inhibiting calcification and OB gene expression. Our results also demonstrate that the observed effects of ferritin are not due to alterations of Pi uptake, and the decrease in the level of intracellular Pi that results from supplementation with ferric iron can be explained by its Pi-binding capacity.

In this study we demonstrate that apoferritin dose-dependently decreases the expression of Cbfa-1. However, it must be noted that this may not be a direct and sole explanation for the observed effects, and there may be other mechanisms responsible for such downregulation that must be elucidated by future studies. In the iron oxidation catalyzed by the

ferritin/ferroxidase center, ferrous iron reacts with dioxygen to generate hydrogen peroxide and ferric iron (110). The hydrogen peroxide is produced inside the ferritin shell, and most of it is used by the ferroxidase center to oxidize iron (53). However, some of the hydrogen peroxide could escape signal directly via oxidative regulation of regulatory thiols in molecules with signal-transducing phosphatases (54). Indeed, depending on cell types, exogenous hydrogen peroxide or lipid hydroperoxides can either increase (vascular smooth muscle cells) (111-113) or decrease (osteoblast) (111, 112) the elaboration of osteogenic gene regulatory programs. The hydrogen peroxide generated by the ferroxidase activity is one of the candidates that might contribute to the suppression of mineralization and OB maturation provided by ferritin. The lack of any effect of catalase on the OB calcification inhibition by ferritin and the inhibitory effect of ferritin on calcification and osteoblastic differentiation of SMC that we have described above suggest that hydrogen peroxide is not a major mediator in suppression of mineralization and OB maturation provided by ferritin.

This study focused mainly on providing more insight into iron overload and its association with decreasing OB activity, bone deposition, and subsequent osteopenia and osteoporosis. Our findings for the first time explain that iron as a risk factor for decreased osteogenesis mainly exerts its inhibitory actions via upregulation of ferritin, and we suggest that ferroxidase activity of ferritin is crucial in suppressing mineralization and OB maturation. It should be noted that future *in vivo* experiments must be carried out to validate these current *in vitro* findings. These findings will expand the knowledge of both osteogenesis and pathogenesis of iron caused bone defects, and by exposing ferritin as the cause of decreased OB maturation may offer new understanding into better planning strategies to prevent or reverse iron-induced osteoporosis and osteopenia.

The dose of different molecules used in this study were based on preliminary results and based on previous reports (52, 114, 115). In addition, a cytotoxicity assay was performed that did not demonstrate any significant toxicity at the doses used in this study. Furthermore, note

that significant inhibitory effects of heme were observed at 25μm (Figure 1). It has to be noted that levels of circulating heme are usually kept at minimal by several mechanisms including rapid induction of HO-1 and proteins such as hemopexin and hemojuvelin. In contrast during hemolysis, tissue and cellular injury levels of heme markedly elevate secondary to release of heme from hemoglobin, myoglobin, cytochromes and other heme containing proteins. This in turn leads to equimolar elevation of biliverdin, iron and bilirubin. Iron in turn will strongly induce expression of ferritin. The high levels of heme in the vasculature are previously reported (116). It should be noted however that iron levels in the bone are understandably high given the fact the bone marrow is a constant machinery responsible for production of hematopoetic cell lineages. While increased level of iron in the bone and its deleterious effects have been demonstrated by Prussian blue stating, it would be very difficult to measure free iron levels as they are rapidly sequestered by proteins such as ferritin in vivo.

In regards to the first study, it should be noted that different molecules were used to provide mechanistic insight that revealed iron induced upregulation of ferritin prevents smooth muscle transition into osteoblasts. The normal range of circulating iron is close to 30μm/L, levels that would provide marked elevation of intracellular ferritin induction. Moreover, the highlight of the first study is based on profound functional iron deficiency that occurs in inflammatory conditions such as advanced CKD that provides the basis of the hypothesis that iron supplementation could be utilized not only to alleviate anemia but also prevent vascular calcification. The levels of iron used in second study are also similar and would closely represent circulating levels of iron in conditions of iron overload and hemochromatosis.

SUMMARY

The studies that are presented in this thesis have focused on two different, yet overlapping clinical conditions. First, our findings have focused to provide molecular understanding into the mechanism of inhibition of high Pi induced vascular calcification. Our results suggest that induction of ferritin is a potent inhibitor of SMC calcification and transdifferentiation into osteoblast like cells. Second, we sought to investigate the mechanism of iron overload induced osteoporosis. We provide evidence that iron inhibits osteoblast activity via upregulation of intracellular ferritin. In both studies we found that ferroxidase activity plays a central role in inhibition of vascular calcification and osteoblast activity. These studies, for the first time, provide mechanistic insight into two very detrimental clinical conditions and hence offer novel avenues of understanding and future research that may lead to innovative therapeutic modalities.

A relationship between calcification and iron metabolism has never been explored, although it should be noted that most patients who have CKD and require renal replacement therapy have vascular calcification and deranged iron homeostasis. Moreover, in patients with CKD, there is an accumulation of iron in reticuloendothelial cells that is accompanied by higher levels of plasma ferritin; however, this increase largely results because most of such iron is sequestered by reticuloendothelial cells and its availability to other cells is significantly reduced. This translates to depletion of intracellular ferritin and subsequent anemia of chronic disease. In inflammatory diseases such as CKD, cytokines released by activated leukocytes and other cells exert multiple effects. These contribute to the reduction in hemoglobin levels and increased hepatic synthesis of hepcidin that in turn binds to ferroportin, the transporter that allows egress of iron from reticuloendothelial macrophages and from intestinal epithelial cells. Binding of hepcidin leads to internalization and degradation of ferroportin. The corresponding

sequestration of iron within the macrophages limits iron availability to all cells. On the basis of our observations, we suggest that such derangements in iron metabolism may facilitate Pi-induced vascular calcification; therefore, parenteral iron administration may be considered not only to replete iron and correct anemia but also to prevent vascular calcification via increasing intracellular ferritin expression and decreasing extracellular Pi level, especially when the inflammation is well controlled.

The latter part of this thesis revealed seminal findings that identify ferritin induction by iron overload to be the mechanism that decreases osteoblast activity subsequently leading to osteopenia and osteoporosis. Therefore, These findings will expand the knowledge of both osteogenesis and pathogenesis of iron caused bone defects, and by exposing ferritin as the cause of decreased osteoblast maturation may offer new understanding into better planning strategies to prevent or reverse iron-induced osteoporosis and osteopenia.

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List of publications related to the dissertation

1. **Zarjou, A.**, Jeney, V., Arosio, P., Poli, M., Zavaczki, E., Balla, G., Balla, J.: Ferritin ferroxidase activity: A potent inhibitor of osteoenesis.
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