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Correction of the mineralization defect in hyp mice treated with protease inhibitors CA074 and pepstatin

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Abstract

Increased expression of several osteoblastic proteases and MEPE (a bone matrix protein) occurs in X-linked hypophosphatemic rickets (hyp). This is associated with an increased release of a protease-resistant MEPE peptide (ASARM peptide), a potent inhibitor of mineralization. Cathepsin B cleaves MEPE releasing ASARM peptide and hyp osteoblast/osteocyte cells hypersecrete cathepsin D, an activator of cathepsin B. Our aims were to determine whether cathepsin inhibitors correct the mineralization defect in vivo and whether hyp-bone ASARM peptide levels are reduced after protease treatment. Normal littermates and hyp mice ($n = 6$) were injected intraperitoneally once a day for 4 weeks with pepstatin, CA074 or vehicle. Animals were then sacrificed and bones plus serum removed for comprehensive analysis. All hyp mice groups (treated and untreated) remained hypophosphatemic with serum 1,25 vitamin D3 inappropriately normal. Serum PTH was significantly elevated in all hyp mice groups relative to normal mice ($P = 0.0017$). Untreated hyp mice had six-fold elevated levels of serum alkaline-phosphatase and two-fold elevated levels of ASARM peptides relative to normal mice ($P < 0.001$). In contrast, serum alkaline phosphatase and serum ASARM peptides were significantly reduced (normalized) in hyp mice treated with CA074 or pepstatin. Serum FGF23 levels remained high in all hyp animal groups ($P < 0.0001$). Hyp mice treated with protease inhibitors showed dramatic reductions in unmineralized osteoid (femurs) compared to control hyp mice (Goldner staining). Also, hyp animals treated with protease inhibitors showed marked and significant improvements in growth plate width (42%), osteoid thickness (40%) and cortical area (40%) ($P < 0.002$). The mineralization apposition rate, bone formation rate and mineralization surface were normalized by protease-treatment. High-resolution pQCT mineral histomorphometry measurements and uCT also confirmed a marked mineralization improvement. Finally, the growth plate and cortical bone of hyp femurs contained a massive accumulation of osteoblast-derived ASARM peptide(s) that was reduced in hyp animals treated with CA074 or pepstatin. This study confirms in vivo administration of cathepsin inhibitors improves bone mineralization in hyp mice. This may be due

to a protease inhibitor mediated decrease in proteolytic degradation of the extracellular matrix and a reduced release of ASARM peptides (potent mineralization inhibitors).

Keywords

MEPE; Rickets; Osteomalacia; PHEX; Mineralization; FGF23

Introduction

A mutation in the PHEX gene (a zn-metalloendopeptidase) is primarily responsible for X-linked hypophosphatemic rickets (hyp) [29,37,70,73]. The disease is characterized by low serum phosphorus, disregulated or inappropriately normal serum 1,25 vitamin D3, elevated serum alkaline phosphatase and severe hypomineralization defects of bone and teeth leading to rickets and osteomalacia [18,66,67]. Excess production of MEPE a bone-tooth matrix protein and FGF23 (a phosphaturic factor or phosphatonin) also occurs as a consequence of PheX inactivation in hyp [2,7,32,40,46,47,82,86,88]. This and other data implicate these molecules as potential downstream factors for the pathogenesis of the renal and skeletal abnormalities. Although the physiological or pathophysiological pathway(s) that link PHEX, FGF23 and MEPE still remain to be determined, evidence overwhelmingly suggests that excess full-length FGF23 is responsible for directly or indirectly mediating the hypophosphatemia and reduced serum 1,25 vitamin D3 in HYP [38,78,83,88]. Also, MEPE inhibits phosphate uptake and mineralization in vivo and in vitro [17,69,71] and is involved in extracellular matrix mineralization. Thus, MEPE might be a component part of a parallel or contiguous FGF23 hyp pathophysiological pathway.

After cloning the PHEX gene with the HYP consortium [29,37,70,73], we directed our efforts to characterizing potential PHEX substrates, co-activators or ligands. To this end, we cloned and identified MEPE as a completely novel extracellular matrix protein from an oncogenic hypophosphatemic tumor [68,72]. We mapped the gene (MEPE) to chromosome 4q21.1 amidst a cluster of bone-dentin salivary proteins with shared features [68]. Following the cloning of human MEPE, rat and mouse mepe were also cloned [1,62]. Osteoblasts derived from mice lacking mepe have increased mineralization ex vivo and the mice have increased bone mass with a resistance to aging-associated trabecular bone loss, consistent with MEPE functioning as a negative regulator of extracellular matrix mineralization and bone-formation [30,45]. We also have evidence that MEPE's ability to impair mineralization involves proteolytic processing leading to the production of ASARM peptide(s) and that PHEX may regulate this process [7,17,32,67,69,71]. In this regard, we have since determined that MEPE binds to PHEX via the PHEX zn-binding motif and a carboxy terminal MEPE ASARM motif (Acidic Serine Aspartate-Rich MEPE associated motif) [69]. The ASARM motif when liberated as a free phosphorylated peptide (2.2 kDa) is a potent inhibitor of mineralization and phosphate uptake [7,17,32,67,69,71]. The motif also binds to PHEX with high affinity and may interfere with PHEX activity [69]. Of note, PHEX protects MEPE from cathepsin proteolysis [32], and hyp osteoblasts overexpress proteases (cathepsin D, NEP, ECEL1) and MEPE [1,2,7,16,21, 31,32,40–42,46,51,67,68,74,77]. Since the ASARM motif is resistant to proteolysis, overproduction of MEPE protein in the presence of excess proteases would be expected to generate large amounts of biologically active protease-resistant ASARM peptide(s) [67–69,71]. Also, excess protease production may result in inappropriate degradation of important and related extracellular matrix proteins (DMP-1 for example) that play clear roles in promoting mineralization and also contain ASARM motifs [44,87]. In confirmation of this, we and others have measured excess circulating levels of either COOH-terminal MEPE or ASARM peptides in HYP and OHO patients [7,40,67]. We have also confirmed that hyp mice kidneys have excess levels

of ASARM peptide epitopes in regions consistent with the proximal convoluted tubules [7]. Also, MEPE is expressed in osteocytes/osteoblasts and in the proximal convoluted tubules of the kidney (the site of the renal defect in hyp) [7,59]. Moreover, MEPE protein circulates in serum and the levels correlate with serum phosphate, PTH and BMD in an age-dependent manner [39].

Since the observed overexpression of proteases and MEPE might be responsible for the noted increased release of protease-resistant MEPE ASARM peptides in hyp, we resolved to determine whether bolus administration of protease inhibitors (pepstatin and CA074) could transiently reverse this. Our experiments confirmed that in vivo administration of pepstatin (a non-specific cathepsin D inhibitor) or CA074 (a specific cathepsin B inhibitor) markedly improved the defective mineralization phenotype in hyp mice without correcting the elevated FGF23 levels, hypophosphatemia and abnormal 1,25 (OH)D levels. The related finding that levels of circulating ASARM peptides were reduced in protease inhibitor-treated hyp mice support a direct role of excess ASARM peptides in the hyp hypomineralization defect [67,69,71]. However, as the mineralization defect was markedly improved but not completely cured the serum phosphate levels likely also play an important role. This is because hypophosphatemia still persisted in the hyp protease-treated mice. Thus, these experiments suggest that defective mineralization in hyp may not be solely due to hypophosphatemia.

Materials and methods

Intraperitoneal administration of pepstatin and CA074 into wild-type and hyp mice

Wild-type (W) or hyp (H) male mice (12 weeks old, C57-BL6) were injected intraperitoneally with either vehicle (V), pepstatin (P) or CA074 (C) as follows: (1) WV (wild type with vehicle), (2) WP (wild type with pepstatin), (3) WC (wild type with CA074); (4) HV (hyp with vehicle), (5) HP (hyp with pepstatin), and (6) HC (hyp with CA074). The injections were given as a daily bolus 5 days a week for 4 weeks. Both protease inhibitors were prepared in vehicle (0.9% NaCl, containing also (v/v) 10% ETOH and 0.5% DMSO) and each bolus (200 μ l) consisted of 50 μ g pepstatin or 100 μ g CA074. Mice tolerated the bolus injections at indicated dosages and intervals well without any sign of discomfort or sickness. For dynamic histomorphometry measurements, two single tetracycline injections (20 mg/kg) were given 5 days apart prior to sacrifice. Blood samples were collected retro-orbitally under anesthesia and serum prepared as described previously [14]. All bones were then removed cleaned of tissue and fixed in Mellonig's modified phosphate-buffered formalin solution (non-decalcified). This was followed by a graduated series of dehydration steps in ethanol 70% to 100% prior to further processing for histology, histomorphometry, pQCT and uCT (see below).

Serum assays

Assays for serum phosphate, calcium, chloride, potassium, creatinine, glucose and alkaline phosphatase were carried out using routine methods and diagnostic kits (Pointe Scientific Inc. Michigan USA) as described previously [71]. Serum 1,25 vitamin D3 levels were estimated using the RIA assay kit from Immunodiagnostic System, Inc. (Baldon, UK), and serum PTH was measured using the mouse intact PTH (1–84) ELISA kit from Immutopics, Inc. (San Clemente, CA). Serum ASARM-peptide levels were measured using an ELISA method that we previously developed and reported with minor modifications [7]. Specifically, instead of a chemiluminescent end-point detection a colorimetric reagent was used (Pierce 1-StepTMUltra TMB-ELISA reagent; 490 nm). Serum FGF-23 levels were measured using a two-site enzyme-linked immunosorbent assay kit (Kainos Laboratories,

Inc. Japan). The FGF-23 ELISA Kit is a two-site enzyme-linked immunosorbent assay kit for the measurement of full-length FGF-23 in serum.

Histology

Proximal ends of tibias were decalcified in EDTA for 2–3 days until complete demineralization [75]. Complete decalcification was checked using Faxitron X-rays. Decalcified tibias were dehydrated and embedded in paraffin, after which 5- μ m sections were cut on a rotary microscope.

Histomorphometry

All bone samples for histomorphometry were trimmed to the region of interest, then dehydrated in a series of ascending EtOH concentrations. Following dehydration, bone samples were infiltrated and then embedded undecalcified in a methyl methacrylate-dibutyl phthalate plastic composite [4]. Serial 4- and 8- μ m sections were stained with Goldner's trichrome and left unstained, respectively. Using Goldner's stained slides, two-dimensional parameters of cancellous bone were measured in a 2-mm square, 1 mm distal to the lowest point of the growth plate. Dynamic measurements were made on 8 μ m unstained sections using epifluorescence microscopy. For deriving the dynamic parameters, the fluorochrome labels (tetracycline) were measured and the mineralizing surface (MS), mineral apposition rate (MAR), bone formation rate-surface referent (BFR/BS), and bone formation rate–bone volume referent (BFR/VR) were calculated as previously described [61]. The double label width of cortex and the mineral apposition rate (MAR) was calculated as interlabel width/labeling period. Cancellous bone volume (BV/TV), trabecular measurements, and osteoid volume (OV/BS) were calculated using a digitized image analysis system and Osteomeasure software version 4.00c [52] (Osteometrics, Atlanta, GA) interfaced with a Nikon Eclipse E400 light/epifluorescent microscope and video subsystem. Histomorphometry values, dynamic and static were expressed according to Parfitt et al. [61]. All samples were evaluated in a blinded manner.

Immunohistochemistry

Decalcified bone sections were immunostained with a polyclonal antibody raised against MEPE ASARM-peptide (NH₂-CFSSRRRDDSSSESDSGSSSE-SDGD-COOH) as previously reported [7,66,69,71]. Five-micrometer paraffin-embedded sections were dried overnight on precharged slides (Pre-cleaned Superfrost Plus, VWR Scientific, West Chester, PA). Slides were deparaffinized and rehydrated. Sections were then incubated with 0.03% H₂O₂ for 15 min to block endogenous peroxidase activity, blocked with avidin/biotin blocking solution (Vector Laboratories Inc.) for 30 min and finally a 5% chicken/goat/horse serum solution for 45 min at room temperature. An antigen retrieval method was then applied to the sections using a pressure cooker and citrate buffer 10 mM pH 6 for 10 min. Slides were allowed to cool down for 20 min to room temperature. The sections were then immunostained with the primary polyclonal antibody for 1 h at room temperature. Negative control slides were incubated with rabbit IgG (5 μ g/ml). All sections were then incubated with goat anti-rabbit biotinylated secondary antibody (1/150) (Vector Laboratories Inc.) for 30 min at room temperature and developed using the Vectastain ABC kit (Vector Laboratories Inc.) and stable DAB (Invitrogen Corp.) counterstained with hematoxylin, dehydrated and mounted with Cytoseal XYL (Richard Allen Scientific).

Peripheral quantitative computer tomography (pQCT)

A Stratec XCT-RM was used to perform pQCT scans of the proximal tibia and midshafts of the tibia and femur. All scans were analyzed using a threshold for delineation of external boundary, and an area peel for subdivision into a cortical/subcortical region and a cancellous

subregion. The BMC (g/cm^2), BMD (g/cm^3), and area properties of each subregion were then determined by system software analysis modes (Software Version 5.40). In the metaphysis and diaphysis of both tibias and femurs, a 0.5-mm segment was scanned for images (12% and 50% from the distal end, respectively). The position was verified using scout views and a single 0.5 mm slices perpendicular to the long axis of the tibia shaft was collected. Similarly, 0.5 mm slices of the tibial and femoral midshafts were also obtained.

Micro-computed tomography (uCT)

A SkyScan 1076 micro-computed tomography machine was used to image the proximal tibia and femurs of representative samples from WT, HYP mice, and HYP mice treated with CA074 (Fig. 11). Samples were scanned at 9- μm resolution, and captured images were rendered using machine software.

Statistical methods

Differences were assessed statistically by the use of Newman–Keuls multiple comparison equations after one-way analysis of variance (ANOVA, non-parametric), or, *t* tests (unpaired) with or without Welch correction (two-tailed; 95% confidence interval) as indicated in the legends.

Results

Serum analysis

Serum levels of phosphate were markedly and significantly reduced in hyp mice groups compared to normal animal groups, treated and untreated (Fig. 1A). Addition of pepstatin and/or CA074 had no significant effect on normal or hyp animal serum PO_4 levels (Fig. 1A). Also there were no significant differences in serum Ca or 1,25 vitamin D3 between any of the groups. The values for normal mice were 10.06 mg/dl SEM = 0.6 for serum calcium and 1193 pM for serum 1,25 vitamin D3. The normal levels of 1,25 vitamin D3 in the hyp animals (treated and untreated) were inappropriate as these mice were hypophosphatemic. Serum PTH levels were significantly elevated in all hyp groups compared to normal mice groups (Fig. 1D). Also, bolus administration of pepstatin or CA074 had no effect on PTH levels in normal and hyp mice. As previously reported the levels of serum alkaline phosphatase (a marker of bone turn-over) and ASARM-peptide(s) were elevated six- and three-fold respectively in hyp vehicle-treated animals compared to normal mice [7,40,67]. In contrast, the levels of serum alkaline phosphatase and ASARM-peptide(s) were both dramatically and significantly reduced (effectively normalized with pepstatin) in hyp animals treated with pepstatin and CA074 (Figs. 1B and C). Addition of pepstatin and CA074 had no significant effect on serum alkaline phosphatase or serum ASARM peptides in normal animals. As expected FGF23 serum levels were markedly elevated in hyp vehicle mice relative to the normal mice but were not affected by the addition of pepstatin and CA074 (hyp vehicle = 615.7, SEM = 133.7; hyp-CA074 = 544.8, SEM = 193.8; hyp-pepstatin = 665.5, SEM = 128.6; normal vehicle = 67.00, SEM = 4.509, normal-CA074 = 63.60, SEM = 16.93; normal-pepstatin = 32.00 SEM = 12.10, all units pg/ml).

Peripheral quantitative computer tomography (pQCT)

Significant improvements in bone quality were measured in hyp animals treated with pepstatin and CA074 as assessed using pQCT. Figs. 2A to D present in histogram form key representative measurements from femurs and tibiae that illustrate the improvements in hyp mouse bone after pepstatin and CA074 treatment. Specifically, the femoral diaphyseal mean density of pure cortical bone (CRT_DEN) and the mean density of total bone (TOT_DEN) were significantly improved (Fig. 2A and B). Similarly, tibial diaphyseal cortical thickness

as measured using the circular ring model (CRT_THK_C) and cortical area assigned to be pure cortical (CRT_A) were also significantly improved (Figs. 2C and D). Improvements in tibial diaphyseal total bone areas were also noted with pepstatin and CA074-treated mice ($P = 0.0351$ and 30% improvement data not graphically shown). Further illustrations of the improvements are illustrated in a cross-sectional pQCT epiphyseal femur scan in Fig. 3. The hyp vehicle bone is clearly abnormal with prominent intracortical regions of hypomineralized osteoid as depicted by the red-color (compare Figs. 3A and B). In contrast the normal animal cortical region is predominantly white, and, as defined by the graduated color-scale the cortical bone is mostly mineralized. The representative CA074 and pepstatin-treated hyp mice femurs show clear improvements and major reductions in hyperosteoidosis compared to hyp vehicle mice (compare Fig. 3B with C and D). Of note is the patella region where in normal and hyp animals the degree of hypermineralization is more pronounced. In treated hyp animals the improvement in mineralization status recedes towards this patellar region (perhaps reflecting the increased load and bone turn-over of the patella region in normal and hyp animals).

Histomorphometry

Static—Histomorphometry also confirmed marked improvement in bone with hyp mice treated with either CA074 or pepstatin. Notably, there were significant improvements (decrease in size) in tibia osteoid thickness, cortical area and growth plate width in treated hyp animals compared to hyp vehicle animals (Figs. 4A, B and C). The pepstatin-treated hyp mice presented with the best improvements compared to hyp vehicle mice. There were no significant differences between normal vehicle mice and normal protease inhibitor-treated mice. The decrease in size of the above “histomorphometric” parameters contrasted with an “inverse” symmetrical increase in “pQCT” measurements of mean density of pure cortical bone (CRT_DEN), mean density of total bone (TOT_DEN), cortical thickness (CRT_THK_C), cortical area (CRT_A) in hyp mice treated with protease inhibitors (Figs. 2A to D). This confirms an improvement of mineralization status in treated hyp animals. Specifically the pQCT measurements assess mineralized bone and the histomorphometric measurements include unmineralized osteoid. Thus, pQCT numerical indices are expected to increase and histomorphometric indices decrease in hyp animals with improved mineralization status.

Dynamic—Double tetracycline labeling was used to provide dynamic histomorphometry measurements. Due to severe hyperosteoidosis in the vehicle-treated hyp animal, the labeling was diffuse with considerable quenching (compare Figs. 5A and B). However, in the normal mice (treated and untreated) and in the protease inhibitor-treated hyp mice, the fluorescent tetracycline labels (representing the two injection time points) were clearly resolved (compare Fig. 5B with C and D). Also, in the protease inhibitor-treated mice the label corresponding to the first intraperitoneal injection was less resolved and more diffuse than the second (Fig. 5C). This was likely due to the cumulative exposure of the treated hyp mice to protease inhibitors and thus progressive mineralization improvement. The dynamic recovery of the bone defects in pepstatin and CA074-treated mice were also reflected by changes in the mineralization apposition rate, bone formation rate/bone surface referent and mineralizing surface as a percentage of bone surface for both endocortical and periosteal surfaces (Fig. 6). For each parameter, the hyp mice treated with CA074 or pepstatin were not significantly different to normal animals (treated and untreated) and showed major improvements compared to hyp vehicle animals.

Histology and Goldner stain

Goldner staining also confirmed the above findings (Fig. 7). In Fig. 7, extensive hypomineralized regions (red color and striations) can be seen in the representative hyp-

mouse tibia (Fig. 7B). This contrasts sharply with the blue mineralized color of the normal vehicle-mouse tibia (Fig. 7A). In animals treated with CA074 or pepstatin, a considerable condensation of the red osteoid zones can be seen. Also, these contracted zones form small but distinctive spherical shapes contrasting with the large lamellar red striations of the hyp vehicle control mice (Figs. 7C and D). The histomorphological quantitation of the osteoid regions (see Fig. 4) confirms the reduced osteoid content in the treated hyp mice.

Immunohistochemistry ASARM peptides and Cathepsin D

Cross-sections of tibial cortical-bone stained positive for ASARM polyclonals in all mice (Fig. 8). The brown positive staining extended in diffuse form into the surrounding bone matrix. However, in hyp mice treated with vehicle, the staining was extensive and clearly more pronounced than the normal mice. Also, this over abundant staining was closely associated with the hypomineralized regions of the hyp mouse cortical bone as depicted by the Goldner histological red staining (compare Fig. 8B/C ASARM immunostain with Goldner stain). More specifically, the diffuse ASARM epitope brown staining was associated with embedded osteocytes and osteoblasts, cells known to be specific for MEPE expression. Of additional note are the associations of staining intensity and the levels of circulating ASARM peptide(s) determined by ELISA (Figs. 9A to E). Specifically, the level of cortical bone ASARM immunohistochemical staining is reduced markedly in animals treated with protease inhibitors and the serum levels of ASARM peptides mirrors this pattern (Fig. 9). Cathepsin D distribution in normal and *hyp* mouse femur bones and kidneys were examined by immunohistochemical staining using anti-cathepsin D antibody (Zymed Laboratories, Inc., San Francisco, CA) (Fig. 10). With normal mouse bones, cathepsin D immunoreactivity was detected only occasionally in bone marrow cells, cells lining the endosteal surface and osteocytes. With *hyp* mouse bones, abundant cathepsin D immunoreactivity was detected in numerous bone marrow cells, cells lining the endosteal surface and osteocytes. In addition, prominent cathepsin D immunoreactivity was also detected in the extracellular bone matrix area of *hyp* mouse bone. The hyp cathepsin D immunoreactivity profile closely parallels the ASARM peptide distribution shown in Figs. 8 and 9. This suggests that this protease may play a role in hyp by degrading matrix proteins (MEPE, DMP-1, etc.) and generating free ASARM peptides.

Micro-computed tomography (uCT)

Fig. 11 shows representative uCT scans of normal mouse (vehicle), hyp mouse (vehicle) and hyp mouse (CA074-treated) tibiae. Similar results were obtained with femurs. Compared to wild-type tibia, hyp mice tibia have clear morphological abnormalities that include widened epiphysis, disruption of the growth plate and large fissures that are likely occupied by unmineralized osteoid in the living animal (compare Fig. 11A with B). In contrast, the treated hyp mice tibiae (CA074 and pepstatin) show a clear and marked improvements and the hyp-treated bone compares favorably with the normal mouse tibia (compare Figs. 11C and D with Figs. 11A and B). The improvements likely reflect an increase in mineralization and a reduction in osteoid in the protease-treated hyp animals. Fig. 12 proposes a model to explain the protease inhibitor mediated improved bone phenotype in hyp mice (see also Discussion).

Discussion

The data presented in this study demonstrate a correction of the hyp mineralization defect in hyp mice treated with protease inhibitors CA074 and pepstatin. This observation provides clues to possible bone phenotype mechanisms in hyp and is supportive of the ASARM model [67–69,71]. We would propose that overexpression of osteoblast or osteocyte specific hyp proteases such as cathepsin D, endothelin-converting enzyme-like endopeptidase

(ECE1/DINE), neprilysin [21,41,42,51], may be responsible for the release of a diffusible protease-resistant peptide (ASARM peptide) that inhibits mineralization [66,67,69,71]. Also, the increased osteoblastic protease activity may degrade the extracellular matrix and destroy key proteins required for mineralization. Of direct relevance is a key matrix protein DMP-1 that is related to MEPE (SIBLING proteins). Specifically, DMP-1 null mutant mice have severe mineralization defects reminiscent of osteomalacia and rickets [44,87]. This important matrix protein (DMP-1) also has an ASARM motif in the same carboxy region as MEPE [67,68]. In support of this model, the hyp osteoblast carries an intrinsic mineralization defect [22–26,34,63–65,76,84,85]. For example, heterotransplantation of hyp osteoblasts or hyp-perisotea (intramuscularly) into normal mice does not correct the hyp osteoblast mineralization defect [22,24–26]. Also, co-culture of hyp osteoblast cultures with normal osteoblast cultures or addition of hyp osteoblast conditioned media to normal osteoblasts results in a diffusible factor that inhibits mineralization of the normal osteoblasts [21,41,42,51,56,84]. Addition of protease inhibitors (pepstatin or phosphoramidon) corrects the in vitro mineralization defect in calvarial hyp osteoblast cultures and the production of an inhibitor of renal P (i) uptake [21,51]. Increased levels of ASARM peptides and C-terminal peptide epitopes of MEPE occur in both hyp and TIO [7,40,67]. Also, MEPE and ASARM peptides inhibit mineralization and are phosphaturic in vivo and in vitro [17,67,69,71]. Of note, sera from normal subjects also contain circulating MEPE and the levels correlate with serum PO₄, PTH, and BMD at the neck and the femur [7,39].

The hyp osteoblast and osteocyte clearly have abnormal expression profiles with regard to MEPE and cathepsin D expression. In normal mice, MEPE is expressed predominantly in “mature osteoblasts and osteocytes” and at lower levels in “normal osteoblasts”. In contrast, hyp mice osteoblasts have markedly elevated levels of MEPE expression and also ASARM peptides. Our results demonstrate that the overexpression of cathepsin D in the surrounding bone matrix parallels the ASARM peptide expression profile (Figs. 8, 9 and 10). The cathepsin D immunohistochemistry results add to and confirm our previous studies that also showed cathepsin D activity and protein levels are greatly increased in hyp mouse osteoblast cells [41,42,51]. In our previous studies, we also found that addition of pepstatin in vitro abolished the demineralization effect of the conditioned media derived from hyp mouse osteoblast cells, raising the possibility that cathepsin D may contribute to the mineralization defect in hyp mouse in vivo [41,42,51]. Also, cathepsin D is known to activate cathepsin B, both directly [57] and indirectly through inactivation of cysteine protease inhibitor [43]. Thus, our experiments were aimed at determining whether proteolytic inhibition of either of these proteases prevents or significantly reduces release of ASARM peptides and/or improves the bone phenotype in hyp mice.

Our data do indeed confirm that addition of CA074 (specific cathepsin B inhibitor) or pepstatin (less-specific cathepsin D inhibitor) significantly improves the in vivo mineralization defect in hyp mice. Also, protease inhibitor-treated hyp mice have a significantly decreased (corrected) ASARM peptide serum levels and serum alkaline phosphatase activity (SAP). Of further relevance to the model, we have reported previously that PHEX protects MEPE from cathepsin B proteolysis and more importantly release of the protease-resistant ASARM peptide [32,67]. PHEX binds non-proteolytically to MEPE as measured using surface plasmon resonance studies [69]. The interaction involves the PHEX catalytic binding site (zn-motif) and the COOH-terminal ASARM motif of MEPE. PHEX therefore protects MEPE from cathepsin B proteolysis in vitro (and thus release of MEPE ASARM peptide) by binding directly to MEPE via the MEPE ASARM motif [67,69]. Although the binding of PHEX to MEPE may serve to protect MEPE from proteolysis, we cannot exclude the possibility that MEPE either activates or inhibits PHEX activity in vivo. This is not unprecedented as MEPE SIBLING protein family members DMP-1, osteopontin and BSP all have specific zn-metalloen-dopeptidase MMP binding partners [28]. Bone

sialoprotein specifically binds proMMP-2 and active MMP-2, while osteopontin binds proMMP-3 and active MMP-3, and dentin matrix protein-1 binds proMMP-9 and active MMP-9. The direct binding of the respective MMPs to their partner SIBLING activates the pro- or mature form of the enzyme directly [28,59].

Of note is the recent finding of specific and localized expression (protein and mRNA) of MEPE in the proximal convoluted tubules (PCT) of the kidney [59]. This expression is confined to the luminal or brush border region that is the site of the Na-dependent phosphate co-transporter (NPT2) defect in HYP/hyp. Recently, we measured greatly increased levels of ASARM-peptide epitope in the PCT of hyp mice kidneys [7]. Also, as mentioned earlier, MEPE protein circulates in normal human serum (up to 1.5 µg/ml) and the levels correlate with serum phosphate, 1,25 vitamin D3 and bone mineral density [39]. MEPE is also phosphaturic when administered as a bolus or infusion in vitro and in vivo [17,67,69,71]. Thus, although our studies are primarily focused on correction of the mineralization defect and bone phenotype in hyp mice, our serum phosphate and vitamin D observations are also worthy of comment. Specifically, in hyp animals treated with pepstatin or CA074 the mineralization defect improves dramatically even though the hypophosphatemia persists. Also, the levels of 1,25 vitamin D3 remained inappropriately normal in all hyp mice groups (treated and untreated), and the levels of FGF23 also remained high. It is likely that the persistent hypophosphatemia in treated mice was due to the interplay of additional phosphaturic factors (FGF23, sFRP4 or FGF7; [6,9,38,88]) unaffected by CA074 or pepstatin. Thus, although the ASARM peptide is phosphaturic when administered to normal animals, it may well play a peripheral or component role in the hypophosphatemia in hyp mice. This is because of the continued presence and elevation of other potent phosphatonins like FGF23. In support of this the levels of FGF23 remained high for vehicle-treated and protease inhibitor untreated hyp mice in our study (see Results section for levels; serum analysis). Thus, correcting or reducing elevated ASARM peptide levels in hyp is expected to have only a marginal effect on serum phosphate.

A phosphate/mineralization disconnect is not unprecedented, and there are extensive experimental and clinical evidence for persistent rickets and osteomalacia in the presence of elevated or normalized phosphate in mice and humans. In hyp mice and in humans with X-linked rickets, the mineralization defect is not corrected by dietary phosphate supplementation but also requires supra-physiological doses of calcitriol [8,11,14,15,49,50,60]. Indeed, early and classic studies with hyp mice and human patients were able to show that although dietary phosphate supplementation corrects rickets and the endochondral calcification defect, it does not correct the endosteal mineralization defect [19,20,35,48–50]. Also, the dentinal mineralization defects in the hyp mouse are recently reported as due to intrinsic odontoblast defects and independent of serum phosphate levels [58]. Intriguingly, more recent findings using null-mutant mice suggests that the phosphate, calcium, vitamin D mineralization regulatory axis is extraordinarily complex and dependent on physiological and tissue specific factors. For example, null-mutant Na-dependent phosphate co-transporter (NPT2^{-/-}) mice are hypophosphatemic but do not have rickets or osteomalacia. Indeed these animals have an age-dependent overcompensation of the skeletal phenotype [5,13,33]. Also, these mice have a predisposition to develop massive renal stones [13]. Secondary ablation of the 1-alpha-hydroxylase gene to produce a double null-mutant corrects the renal stones defect [79]. Remarkably, in patients with hereditary hypophosphatemic rickets with hypercalciuria (HHRH), the systemic abnormalities mirror the NPT2 null-mutant mice except for the bone phenotype [80,81]. Specifically, the patients are hypophosphatemic with elevated 1,25 vitamin D3, high serum calcium but in contrast to the NPT2 null-mutant mice do have rickets. Recently, attempts to rescue hyp mice using a phex transgene driven by a β-globin promoter almost completely corrected the defective

mineralization phenotype (rickets), but the hypophosphatemia persisted. Again this suggests a physiological de-coupling of mineralization and serum phosphate [3,27].

Extensive ex vivo and in vitro experiments also suggest a phosphate mineralization disconnect. For example, heterotransplantation of hyp mice periosteal/osteoblasts into normal mice does not correct the abnormal mineralization phenotype in vivo [22–25]. This is true also for hyp mice supplemented with enriched phosphate and 1,25 vitamin D₃ diets prior to removal and heterotransplantation of bone cells to normal mice [25,26]. Isolated hyp-osteoblasts also have additional diverse defects ex-vivo that are not corrected with phosphate supplementation [10,12,34,36,53,54,63–65]. Thus, these extensive studies and the studies described in this paper “support” the notion that defective mineralization is not solely due to hypophosphatemia. More recently, the phosphate mineralization axis has acquired an additional order of complexity as demonstrated by elegant experiments carried out by Murshed et al. [55]. From these experiments and others, the exquisite importance of the ratios between phosphate (Pi) and pyrophosphate (PPi) and expression of the genes regulating this have become apparent. In this regard, although our experiments confirm a marked improvement in the hyp mouse mineralization defect after protease inhibitor treatment, we were unable to “completely” correct the abnormality. Therefore, we cannot exclude the possibility that the persistent hypophosphatemia in our experimental system prevented a complete remission of the mineralization defect.

Fig. 12 illustrates a speculative model for the mineralization improvements we noted in hyp mice treated with CA074 and pepstatin. Administration of protease inhibitors to hyp mice is proposed to reduce degradation of important extracellular proteins required to foster mineralization (minfostins) and to reduce release of protease-resistant ASARM peptides that inhibit mineralization (minhibins). These may include DMP-1 (a putative minfostin) and MEPE (a putative minhibin). MEPE and DMP-1 are SIBLING proteins that contain protease-resistant ASARM-motifs in the same carboxy terminal region of the protein [67,68]. In the case of DMP-1, an intact “ASARM motif” may help promote mineralization whereas inappropriate degradation and release of the small protease-resistant peptide in hyp may inhibit mineralization. The scheme is a working model and speculative. More detailed work needs to be done to clarify a number of molecular aspects. Although it is clear that PHEX protects MEPE from proteolysis and thus release of ASARM peptide (minhibin), we cannot exclude the possibility that the PHEX-MEPE and or ASARM peptide interaction is required to inactivate or activate PHEX activity. As mentioned earlier, there is precedence for this as DMP1, BSP and osteopontin are able to directly activate specific “partner” Zn-matrix metalloendopeptidases [28,59]. Also, as mentioned, the ASARM peptide is a feature of a number of SIBLING bone-dentin salivary proteins (DMP1, osteopontin, DSPP, statherin etc.) that map to chromosome 4 [67,68]. Thus, these diverse ASARM peptide sources may also play a key role in hyp and in tumor induced osteomalacia (TIO) and share similar epitopes. Of further note, although PHEX-MEPE protein–protein interactions have been characterized and reported [69], PHEX-DMP-1 interactions have not been studied and may yield interesting findings.

In conclusion, the data presented are supportive of the ASARM model of end-point mineralization described earlier [67–69]. The data also support the notion that defective mineralization in hyp is not solely due to hypophosphatemia. Specifically, proteolytic degradation of key matrix proteins required for mineralization (minfostins) and release of protease-resistant ASARM peptides (minhibins) may also play a role. Finally, these experiments confirm that release of ASARM peptides in hyp mice treated with CA074 and pepstatin protease inhibitors is reduced and associated with improved mineralization. More work is needed to confirm whether the ASARM protease molecular model is correct and to

determine whether the use of “protease inhibitors” have clinical applications for bone mineral loss diseases in general.

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Fig. 1.

A graphical illustration of serum PO4 mg/dl (A), alkaline phosphatase U/dl (B), ASARM peptide nM (C) and PTH (1–84) (D): the *X*-axis labels WV, WP, and WC indicate wild-type mice treated with vehicle (WV), pepstatin (WP) and CA074 (WC). Similarly, hyp mice *X*-axis labels represent vehicle-treated (HV), pepstatin-treated (HP) and CA074-treated mice (HC) respectively. The smaller inset histogram attached to graph D represents a pooling of serum PTH (1–84) values for wild-type and hyp mice groups respectively. For statistics, a one-way analysis of variance non-parametric (ANOVA) followed by a Newman–Keuls multiple comparison test was carried out. For further details, see Materials and methods and statistics section.

Fig. 2.

A graphical illustration of peripheral computed tomography analyses (pQCT) for wild-type and treated/untreated hyp mice: X-axis labels are as described for Fig. 1. Mean density of pure cortical bone (CRT_DEN) from the femoral-diaphysis in mg/cm³ (A), mean density of total bone (TOT_DEN) from the femoral diaphysis in mg/cm³ (B), cortical thickness (circular ring model) of the tibial-diaphyseal region using the circular ring model (CRT_THK_C) in millimeter (C), cortical area assigned to be pure cortical bone in the tibial-diaphyseal region (CRT_A) in millimeter squared (D). For each measurement, a marked improvement is observed in hyp mice treated with pepstatin or CA074. Statistical analyses represent unpaired *t* tests with Welch correction (two-tailed; 95% confidence interval).

Fig. 3.

Representative pQCT cross-sectional transverse scans of subepiphyseal femur regions of normal (A), vehicle-treated hyp mouse (B), hyp mouse treated with pepstatin (C), and hyp mouse treated with CA074 (D). No changes were observed between control vehicle normal mice and treated (pepstatin/CA074) normal mice (not shown). A marked improvement can be seen in hyp mice treated with pepstatin or CA074 (compare B with C and D). The upper bar is a graduated color-index representation of the degree of mineralized bone (density mg/cm³). White indicates highly mineralized bone and red to black unmineralized matrix. The annular cortical regions of the normal mouse (A) are white and the marrow space black. In contrast the hyp mouse treated with vehicle contains intracortical regions densely infiltrated with red to orange stipulations indicative of massive hyperosteoidosis or hypomineralized matrix. The colored regions are also markedly diminished in hyp mice treated with pepstatin (D) or CA074 (C). This is indicative of a major improvement in the hyp phenotype mineralization defect.

Fig. 4.

Histomorphometric measurements of cortical-bone osteoid-thickness (μm) at the tibia diaphysis (A), cortical area (mm^2) at the tibia diaphysis (B) and growth plate width (μm) at the tibia epiphysis (C). X-axis labels are as described in Fig. 1. In all cases, a dramatic improvement is observed in hyp mice treated with CA074 or pepstatin. No measurable differences were found between control normal mice and control-treated mice. Statistical analyses represent unpaired *t* tests (two-tailed; 95% confidence interval).

Fig. 5.

Representative fluorescence photomicrographs of tibial cortical bone from normal vehicle-treated mouse (A), vehicle-treated hyp mouse (B) hyp mouse treated with pepstatin (C) and hyp mouse treated with CA074 (D). Mice were injected twice with tetracycline over 3 days as described in Materials and methods. Two distinct lamellar layers can be seen with normal mice treated with vehicle (A) hyp mice treated with CA074 (C) and hyp mice treated with pepstatin (D). There is little binding of tetracycline to control hyp mice cortical bone treated with vehicle (as confirmed by the diffuse quenching and loss of fluorescent layers). This is due to excess osteoid and reduced mineralized bone in hyp mice (B). The improvement in CA074 and pepstatin-treated hyp mice bone can be seen by the distinct lamellar fluorescent layers.

Fig. 6.

Graphical illustrations of dynamic histomorphometry measurements derived from the endocortical surfaces of distal femurs. *X*-axis labels in all graphs are as described for Fig. 1. Mineral apposition rate (MAR) $\mu\text{m}/\text{day}$ (A), bone formation rate/bone surface referent (BFR/BS) $\mu\text{m}^3/\mu\text{m}^2/\text{year}$ (B) and mineralizing surface as a percentage of bone surface (MS/BS)% (C). In all cases, improvements were noted in hyp mice treated with CA074 and pepstatin. Similar results were obtained with periosteal surfaces.

Fig. 7.

Representative photomicrographs of tibial cortical bone stained with histological Goldner stain. Wild-type mouse treated with vehicle (A, WV), hyp mouse treated with vehicle (B, HV), hyp mouse treated with pepstatin (C, HP) and hyp mouse treated with CA074 (D, HC). The blue color represents mineralized-bone and the red color unmineralized osteoid. The wild-type vehicle-treated tibia is predominantly blue and mineralized fully (A). In contrast, hyp mouse cortical bone is infiltrated with massive red striations (red-stained osteoid) and is consequently grossly under-mineralized (B). In marked contrast both pepstatin and CA074 protease inhibitor-treated hyp animals show considerable contraction of the red color and thus a marked improvement in the hyp phenotype mineralization defect (C and D). The immunohistochemistry and Goldner stained regions are matched overlapping $\times 40$ magnifications.

Fig. 8.

Representative photomicrographs of immunohistochemistry slides screened with anti-ASARM polyclonals (cortical region). The lower three photographs ($\times 400$) are screened with either preimmune serum (A) or anti ASARM polyclonals (B and C). Photograph A (preimmune control) is derived from hyp mouse cortical bone. The upper two photographs and middle two photomicrographs are stained with Goldners stain (blue color indicates mineralized bone and red unmineralized osteoid). The boxed rectangles and arrows in the upper two lower magnification pictures ($2\times$) indicate corresponding areas that are magnified and shown in the middle two photographs ($\times 400$). The photographs in column B are normal wild-type mouse and the photographs in column C are derived from hyp mouse bone. There is markedly increased staining for ASARM epitopes (brown color) in hyp mouse bone (bottom photo C) compared to wild-type bone (bottom-photo B). Also, the diffuse brown staining (ASARM epitopes) is associated with the Goldner red-staining unmineralized (osteoid) regions (lower photo C). Specifically with embedded osteocytes and osteoblast like cells. Thus, the ASARM epitope is associated with hypomineralized bone.

Fig. 9.

Representative photomicrographs of tibia-bone cortical sections screened with an anti-ASARM polyclonal. Wild-type mouse treated with vehicle (A, WV), hyp mouse treated with vehicle (B, HV), hyp mouse treated with CA074 (C, HC) and hyp mouse treated with pepstatin (D, HP). Graph E represents serum levels of ASARM peptide and the X-axis labels are as described in Fig. 1. There is intense staining for ASARM epitopes (brown color) in hyp mice treated with vehicle (B). The staining diminishes with hyp mice treated with CA074 and pepstatin (C and D). The lowest staining intensity occurs with normal mice treated with vehicle (A). Serum levels of ASARM peptide mirror the degree of immunohistochemical staining (E).

Fig. 10.

Cathepsin D immunoreactivity in normal and *Hyp* mouse bones. Cathepsin D distribution in normal and hyp mouse femur bones were examined by immunohistochemical staining using anti-cathepsin D antibody. With normal mouse bones (left panel), cathepsin D immunoreactivity was detected only occasionally in bone marrow cells (arrow), cells lining endosteal surface and osteocytes (arrow). With hyp mouse bones (right panel), abundant cathepsin D immunoreactivity was detected in numerous bone marrow cells (arrow), cells lining endosteal surface (arrow) and osteocytes (arrow). In addition, prominent cathepsin D immunoreactivity was also detected in extracellular bone matrix area of *hyp* mouse bone (arrowhead). ($\times 20$).

Fig. 11.

Representative tibial uCT scans of (A) wild type, (B) hyp mouse, (C) hyp-mouse treated with CA074 and (D) hyp-mouse treated with pepstatin. Compared to the wild-type tibia (A) the hyp mouse tibiae (B) has clear morphological abnormalities that include widened epiphysis clear disruption of the growth plate and large fissures that are occupied by unmineralized osteoid in the living animal. In contrast, the CA074 and pepstatin-treated hyp mice tibiae (C and D) show clear and marked improvement that can be compared favorably with the wild-type tibia (A).

Fig. 12.

Schematic illustration of the ASARM-model incorporating the improvements noted in hyp mice treated with protease inhibitors CA074 and pepstatin. We have shown that PHEX binds to MEPE using surface plasmon resonance studies, and this involves the PHEX zn-binding site and the MEPE ASARM-motif (A; [69]). In vitro, this association protects MEPE from cathepsin B cleavage [32,67]. PHEX is mutated in X-linked rickets (hyp) resulting in a loss of PHEX-MEPE binding and consequential increased exposure of MEPE to osteoblastic proteases. Also, MEPE and osteoblastic protease expression/activity (including cathepsin D) is markedly elevated in hyp, and this leads to increased degradation of MEPE and release of protease-resistant ASARM peptide (B; [21,51,67]). Also, degradation of other SIBLING proteins required for mineralization (DMP-1, etc.) may also occur contributing to the increased pool of protease-resistant ASARM peptides. The ASARM peptide is a potent inhibitor of mineralization and thus may play a key role in rickets/osteomalacia. Addition of CA074 (a specific inhibitor of cathepsin B) or pepstatin (a less specific inhibitor of cathepsin D) reduces degradation of MEPE/other SIBLINGs (DMP-1) and thus proteolytic release of protease-resistant ASARM peptides. CA074 does this directly by inhibiting cathepsin B and pepstatin does this indirectly by inhibiting cathepsin D which is known to activate cathepsin B.