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Adipocyte induced arterial calcification is prevented with sodium thiosulfate

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ABSTRACT

Background: Calcification can occur in fat in multiple clinical conditions including in the dermis, breasts and in the abdomen in calciphylaxis. All of these are more common in patients with advanced kidney disease. Clinically, hyperphosphatemia and obesity are risk factors. Thus we tested the hypothesis that adipocytes can calcify in the presence of elevated phosphorus and/or that adipocytes exposed to phosphorus can induce vascular smooth muscle cell (VSMC) calcification.
Methods: 3T3-L1 preadipocytes were induced into mature adipocytes and then treated with media containing high phosphorus. Calcification was assessed biochemically and PCR performed to determine the expression of genes for osteoblast and adipocyte differentiation. Adipocytes were also co-cultured with bovine VSMC to determine paracrine effects, and the efficacy of sodium thiosulfate was determined.
Results: The results demonstrated that high phosphorus induced the calcification of differentiated adipocytes with increased expression of osteopontin, the osteoblast transcription factor Runx2 and decreased expression of adipocyte transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT-enhancer-binding protein α (CEBPα), indicating that high phosphorus led to a phenotypic switch of adipocytes to an osteoblast like phenotype. Sodium thiosulfate, dose dependently decreased adipocyte calcification and inhibited adipocyte induced increase of VSMC calcification. Co-culture studies demonstrated that adipocytes facilitated VSMC calcification partially mediated by changes of secretion of leptin and vascular endothelial growth factor (VEGF) from adipocytes.
Conclusion: High phosphorus induced calcification of mature adipocytes, and adipocytes exposed to elevated phosphorus can induce calcification of VSMC in a paracrine manner. Sodium thiosulfate inhibited this calcification and decreased the secretin of leptin and VEGF from adipocytes. These results suggest that adipocyte exposure to elevated phosphorus may be a pathogenic factor in calcification observed in the skin in calciphylaxis and other diseases.

1. Introduction

Patients with advanced kidney disease such as those with end stage renal disease requiring dialysis are prone to extraskeletal calcification. A lot of attention has been paid to arterial calcification, but other soft tissues are also calcified including the dermis of the skin, breasts and the abdomen [1]. The latter is a common site for calciphylaxis and is characterized by small arteriolar calcification in the dermis with surrounding necrosis and calcification of tissue. Patients often present with livido reticularis that progress to ulcerative lesions with necrotic centers and violaceous borders [1,2]. The disease has a very high mortality rate. Clinically, case controlled studies have demonstrated major risk factors to be hyperphosphatemia, obesity, white race, and the use of Coumadin [3–5]. Treatment with sodium thiosulfate has become common, although the mechanism by which it appears to halt progression of lesions is unknown [6–8]. Studies evaluating vascular calcification in other tissues have found that calcification is the result of an active cellular process mediated in part by upregulation of the osteoblast transcription factor Runx2 to induce a phenotypic switch of vascular smooth muscle cells (VSMC) to an osteochondrocytic phenotype [9]. Given the fact that osteoblasts, adipocytes, and vascular smooth muscle cells have a common mesenchymal stem cell (MSC) origin, we hypothesized that transformation of adipocytes to mineralizing cells may be a cause of calcium deposition in the subcutaneous fat. Alternatively, such transformed cells may potentiate calcification of adjacent arterioles in calciphylaxis.

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2. Materials and methods

2.1. Cell culture

The mouse 3T3-L1 preadipocytic cell line was a gift from Dr. Robert Considine at Indiana University School of Medicine and bovine vascular smooth muscle cells (BVSMC) were isolated from the descending thoracic aorta by the explant method as previously described [10]. Cells were cultured in growth medium consisting of Dulbecco's modified Eagle medium (DMEM, Sigma, St. Louis, MO) and 10% fetal bovine serum (FBS). To induce the 3T3-L1 pre-adipocytes to differentiated adipocytes, confluent 3T3-L1 cells were switched to DMEM with 10% FBS containing 10 μM dexamethasone, 1 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxantine (IBMX) for 7 days [11]. The differentiation of adipocytes was confirmed by the increased expression of adipocyte markers PPARP γ and lipoprotein lipase (LPL) and Oil Red O staining as described below. To induce calcification, 3T3-L1 adipocytes were treated with 5 mmol/l β-glycerophosphate (high phosphorus), 1 U/ml fetal alkaline phosphatase, 10^{-7} mol/l insulin and 50 μg/ml ascorbic acid in the presence of 15% fetal bovine serum [12] for 7 days. In some experiments, adipocytes or BVSMC were treated with various dose of sodium thiosulfate (Sigma, St. Louis, MO).

2.2. Calcium deposition

Cells were decalcified with 0.6 N HCl for 24 h. The calcium content of HCl supernatants was determined colorimetrically by the *o*-cresolphthalein complex one method (Calcium kit; Pointe Scientific) and normalized to protein content as previously described [13].

2.3. Histological analysis

Adipocytes were characterized by Oil Red O (ORO) which stains the lipid droplets in the cytoplasm [11]. Briefly, cells were rinsed in PBS and then fixed in 10% (v/v) neutral buffered formalin. Fixed cells were stained with a working solution of ORO for 30 min at room temperature and counterstained with Harris's hematoxylin for 30 s for nuclear staining. Calcification was determined by Von Kossa staining. Briefly, cells were incubated with 5% silver nitrate solution under ultraviolet light for 60 min. Unreacted silver was removed with 5% sodium thiosulfate for 5 min and counterstained with nuclear fast red for 5 min. Images were collected using a Nikon-inverted microscope with a Nikon D100 digital camera.

2.4. Real time (quantitative) RT-PCR analysis

Total RNA was isolated from 3T3-L1 adipocytes using miRNeasy Mini kit (Qiagen, Valencia, CA). The gene expression in cells was determined by real time PCR using 1 μg of total RNA in TaqMan Reverse Transcription reagent (Applied Biosystems, Foster City, CA). Target-specific PCR primers for Runx2, osteopontin, PPARγ, C/EBPα and LPL were obtained from Applied Biosystems. Real-time PCR amplification was performed using TaqMan Gene Expression Assays (TaqMan MGP probes, FAM dye-labeled) using Applied Biosystems ViiA7 Real-Time PCR system (Applied Biosystems). The cycle number at which the amplification plot crosses the threshold was calculated (C_T), and the $\Delta\Delta C_T$ method was used to analyze the relative changes in gene expression using β-actin as a housekeeping gene [13].

2.5. Co-culture of adipocytes and vascular smooth muscle cells

To determine the paracrine effects of adipocytes and VSMC on each other, co-culture experiments were performed. 3T3-L1 preadipocytes ($0.5 \times 10^4/cm^2$) were first seeded on the 24-well culture plates and induced to differentiate with adipogenic media for 7 days as detailed above. BVSMC ($6 \times 10^4/cm^2$) were seeded in BD Falcon cell culture inserts (0.4 μm, BD Biosciences) with growth media. After reaching confluence, inserts with BVSMC were moved into the wells containing 3T3-L1 adipocytes, with common media but no cell–cell contact. Co-cultures were then incubated with high phosphorus media (containing 5 mmol/l β-glycerophosphate) as detailed above and calcification determined after 3 days. BVSMC in the culture insert alone and adipocyte in culture wells alone were used as controls. In some experiments, co-cultures were incubated in the presence or absence of sodium thiosulfate. To determine the mechanisms of paracrine effects, conditioned media was collected from 3T3-L1 adipocytes treated with control or calcification media and leptin, adiponectin and vascular endothelial growth factor-A (VEGF-A) concentration measured using ELISA kits (R & D systems).

2.6. Statistical analysis

Statistical analysis was conducted by ANOVA. The results are expressed as means ± SD, with $p < 0.05$ considered significant (StatView, SAS Institute, Cary, NC).

3. Results

3.1. High phosphorus induced calcification, increased the expression of osteoblastic genes and decreased the expression of adipogenic genes in 3T3-L1 adipocytes

To determine if high phosphorus induces adipocyte calcification, 3T3-L1 preadipocytes were differentiated and then treated with control or calcification media (high phosphorus) for 1, 3 and 7 days. As shown in Fig. 1, at 7 days the adipocytes in cells treated with control or high phosphorus retained oil red staining (Fig. 1A, top). However, the adipocytes treated with high phosphorus media had increased calcification by histological (Fig. 1A, bottom black area) and biochemical analysis (Fig. 1B). By real time PCR, high phosphorus increased gene expression of the osteoblastic transcription factor Runx2 in adipocytes at day 1 (Fig. 2A) and osteopontin at day 1, 3 and 7 (Fig. 2B). In contrast, in adipocytes treated with high phosphorus, the gene expression of the adipogenic transcription factors PPARγ was decreased at day 3 and 7 (Fig. 2C) and C/EBPα was decreased at day 7 (Fig. 2D). These results suggest that adipocytes exposed to high phosphorus can upregulate osteoblastic transcription factors and downregulate adipogenic transcription factors and facilitate calcification.

3.2. Adipocytes enhance calcification of VSMC in a paracrine manner

To evaluate the potential of adipocytes to affect calcification in BVSMC, 3T3-L1 adipocytes and BVSMC co-cultures were incubated with high phosphorus media and calcification determined after 3 days. The results demonstrated that calcification of BVSMC was significantly enhanced when co-cultured with adipocytes compared to BVSMC alone (Fig. 3A). In contrast, there was no difference in calcification of adipocytes in high phosphorus media incubated alone or co-cultured with BVSMC (Fig. 3B). These co-culture data indicate that adipocytes have the potential to enhance the calcification of VSMC but the reverse was not true.

To determine the mechanism of this paracrine effect, conditioned media were collected and analyzed for factors known to be secreted by adipocytes and in turn known to increase calcification in VSMC. High phosphorus modestly increased VEGF-A secretion from adipocytes compared to control media (5.3 ± 0.2

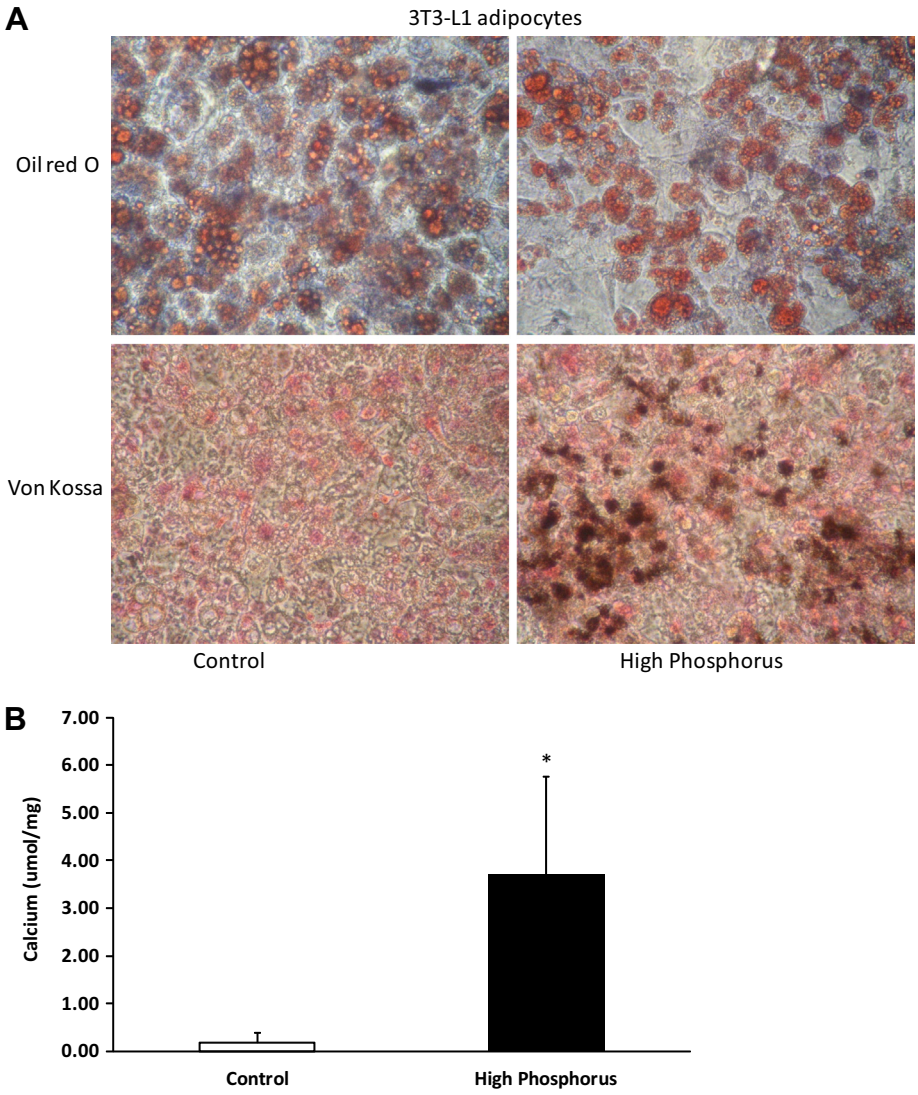

Fig. 1. High phosphorus induced calcification of 3T3-L1 adipocytes: 3T3-L1 pre-adipocytes were cultured in adipogenic media for 7 days to induce differentiation. The differentiated adipocytes were then treated with control or high phosphorus (5 mM β-glycerophosphate) media or control media for 7 days. The cells were stained for Oil Red O as a marker of terminal adipocyte differentiation (A, red stain, top panel) and Von Kossa for calcification (A, black stain, bottom panel). The calcification was also quantified by HCL extraction followed by colorimetric assay (B). The results showed persistence of adipocytes (Oil Red O staining) in high phosphorus conditions (A, top). However, the adipocytes treated with high phosphorus media also had significantly increased calcification by histologic (A, bottom) and biochemical analysis (B). Data are shown as mean ± SD ($n = 9$). $^{*}p < 0.05$, control vs. high phosphorus.

vs. 4.2 ± 0.2 ng/ml, $p < 0.03$). However, there was no significant difference in leptin or adiponectin secretion between control or high phosphorus treated 3T3-L1 adipocytes.

3.3. Sodium thiosulfate inhibited vascular calcification by decreasing secretion of VEGF-A and leptin from adipocytes

We incubated 3T3-L1 adipocytes and BVSMC with high phosphorus media in the presence or absence of various doses of sodium thiosulfate for 3 days and measured calcification. The results show a dose dependent decrease in calcification of adipocytes with sodium thiosulfate. In contrast, the addition of sodium thiosulfate to BVSMC had no effect on calcification (data not shown). We then incubated adipocyte–BVSMC co-cultures with high phosphorus calcification media in the presence or absence of sodium thiosulfate and calcification was determined after 3 days. As shown in Fig. 4, sodium thiosulfate reduced calcification in the co-cultures but had no effect on BVSMC calcification alone (Fig. 4). These results suggest that in addition to inhibition of adipocyte calcification, sodium thiosulfate also prevented adipocyte-mediated enhancement of BVSMC calcification. Furthermore, measurement of secreted factors into the conditioned media demonstrated that, compared to vehicle, sodium thiosulfate treatment significantly decreased VEGF-A secretion (5.30 ± 0.27 vs. 4.05 ± 0.23 ng/ml, $p < 0.001$) and leptin secretion (147 ± 64 vs. 17 ± 6.8 pg/ml, $p < 0.0001$) However, sodium thiosulfate had no effect on adiponectin secretion from adipocytes (6.60 ± 0.23 vs. 6.59 ± 0.18 ng/ml). Cells retained their normal phenotype. These results indicate that sodium thiosulfate directly affects adipocyte calcification, and also prevents the ability of adipocyte to potentiate VSMC calcification, in part by suppression of VEGF-A and leptin secretion from adipocytes.

4. Discussion

Extraskeletal calcification is a common finding in patients with ESRD on autopsy and other studies, with findings pre-dating

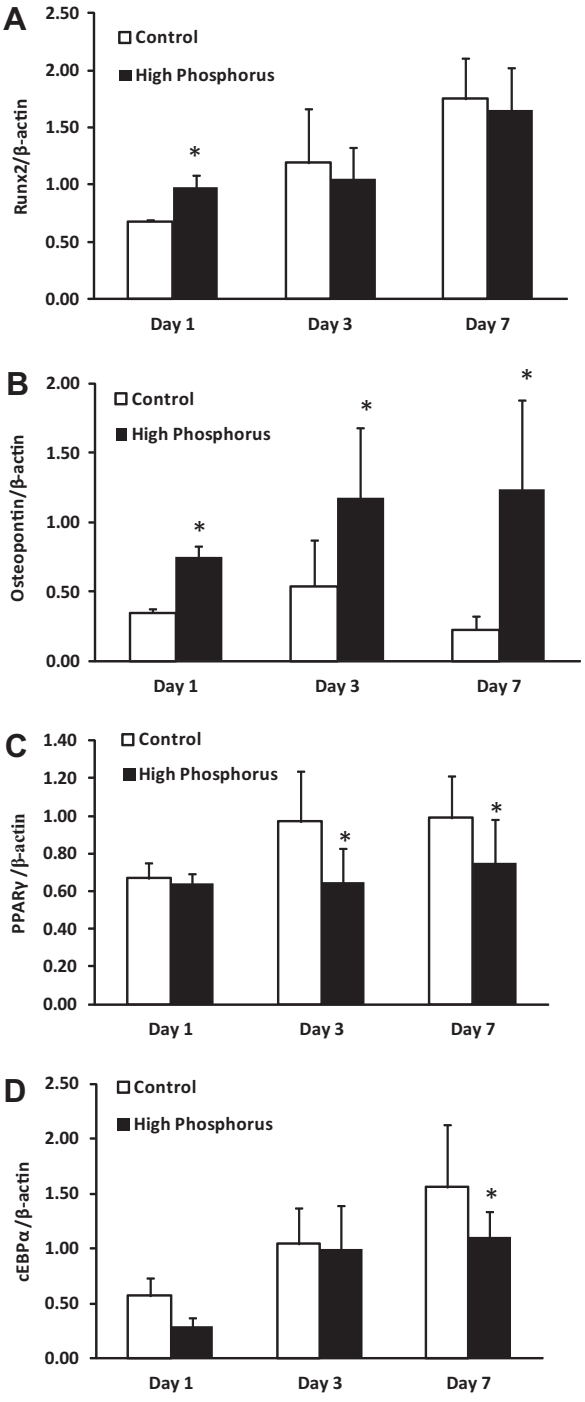

Fig. 2. High phosphorus increased the expression of osteoblastic genes and decreased expression of adipogenic genes in adipocytes. The fully differentiated 3T3-L1 adipocytes were treated with control or high phosphorus media for 1, 3 or 7 days. Total RNA was isolated for real time PCR. High phosphorus increased the expression of osteoblast genes Runx2 in adipocytes at day 1 (A) and osteopontin at day 1, 3 and 7 (B). In contrast, high phosphorus decreased the expression of the adipogenic genes PPARγ at day 3 and 7 (C) and C/EBPα at day 7 (D). Data are shown as mean ± SD ($n = 9$). $^{*}p < 0.05$, control vs. high phosphorus.

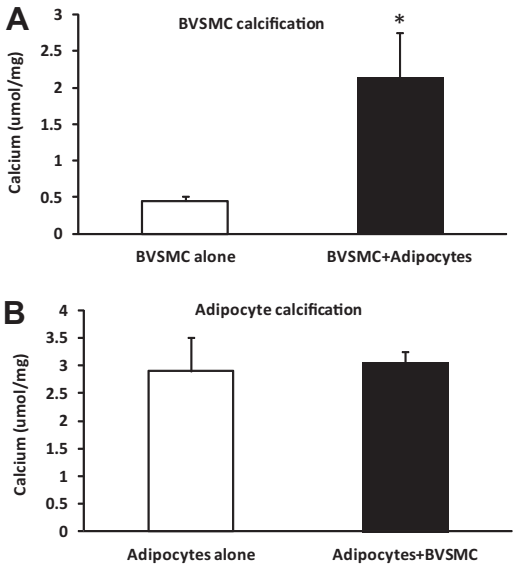

Fig. 3. Adipocytes enhance calcification of bovine vascular smooth muscle cells (BVSMC). 3T3-L1 pre-adipocytes were seeded on 24-well culture plates and induced to adipocyte differentiation with adipogenic media for 7 days. BVSMC were seeded in cell culture inserts with growth media. After reaching confluence, inserts with BVSMC were moved into the wells containing 3T3-L1 adipocyte. Co-cultures were incubated with high phosphorus media and calcification determined after 3 days. Calcification of BVSMC alone (without adipocytes) or calcification of adipocytes alone (without BVSMC) was used as controls. The results demonstrated that calcification of BVSMC was significantly enhanced when co-cultured with adipocytes compared to BVSMC alone (A). In contrast, there was no difference in calcification of adipocytes with or without BVSMC (B). Data are shown as mean ± SD ($n = 9$). $^{*}p < 0.05$, BVSMC along vs. BVSMC with adipocytes.

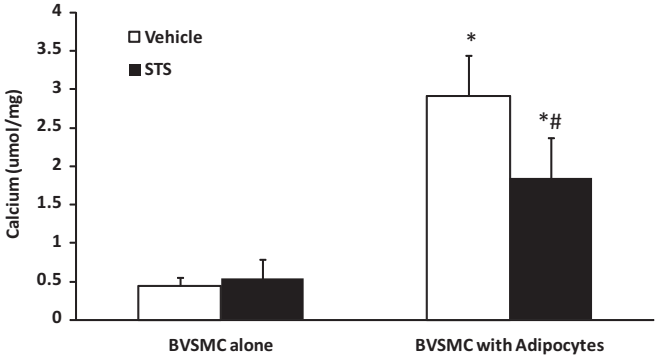

Fig. 4. Sodium thiosulfate (STS) prevented adipocyte enhancement of BVSMC calcification: Fully differentiated 3T3-L1 adipocytes were co-cultured with BVSMC in calcification media in the presence or absence of 50 mM sodium thiosulfate (STS) and calcification determined after 3 days. Calcification of BVSMC alone (without adipocytes) or calcification of adipocytes alone (without BVSMC) was used as controls. In the presence of sodium thiosulfate, the adipocyte enhanced BVSMC calcification was blunted. In contrast, sodium thiosulfate treatment had no effect on BVSMC calcification alone. Data are shown as mean ± SD ($n = 9$). $^{*}p < 0.05$, BVSMC + adipocytes vs. BVSMC alone, with or without sodium thiosulfate; $^{\#}p < 0.05$, sodium thiosulfate vs. vehicle, BVSMC + adipocytes or BVSMC alone.

currently used therapies [14] suggesting abnormalities unique to uremia. Hyperphosphatemia is also a common condition in ESRD patients [15]. Most of the studies examining the pathogenesis of extraskeletal calcification have focused on arterial/vascular calcification. However, calcification can occur in the dermis in the skin, breast, and abdominal fat. The latter is a common location for calciphylaxis, and histologic examination of skin biopsy samples from patients with calciphylaxis reveals vascular calcification in the medial layer of small arterioles (usually 40–50 μm) located predominately in the subcutaneous fat together with surrounding calcification, necrosis and hydroxyapatite deposition in the fat [2,3]. Case control studies have shown that hyperphosphatemia is a clinical risk factor [3,16–18] and many of the therapies currently used (increased dialysis frequency, cinacalcet) lower phosphorus levels [6,19]. In the present study we have demonstrated that adipocytes exposed to high phosphorus media have increased expression of the osteoblast transcription factor Runx2,

increased expression of osteopontin and decreased expression of the adipogenic genes PPARγ and C/EBPα. This pattern of transcription factor expression indicates a phenotypic switch of the cultured adipocytes into an osteochondrocytic cell, further supported by the ability of the adipocytes to calcify. We also demonstrated that adipocytes can induce calcification of VSMC in a paracrine manner.

Osteoblasts and adipocytes have a common origin in mesenchymal stem cells with transcription factor expression driving differentiation [20]. Runx2 is critical for osteoblast differentiation and regulates the expression of osteoblasts; deletion of this gene leads to an absence of mineralized bone and early demise [21]. Adipocyte differentiation is activated by two transcription factors, PPARγ and C/EBP [22]. The increased expression of PPARγ and C/EBP are necessary for the development of fully differentiated adipocytes with the characteristic formation of lipid droplets in the cytoplasm [23]. However, lipid-filled mature adipocytes are generally considered terminally differentiated with a loss of proliferative ability [24]. There appears to be a reciprocal relationship between the regulations of these lineages. For example, in bone marrow derived MSC, there is a progressive increase in adipocyte formation and a decrease in osteoblast number with aging [25]. Human studies confirm these observations, demonstrating that human subcutaneous preadipocytes can differentiate into bone-forming osteoblasts in the presence of high phosphorus [26]. Furthermore, overexpression of Runx2 in 3T3-L1 preadipocytes stimulates transdifferentiation to osteoblasts with increased expression of osteocalcin and alkaline phosphatase activity and downregulation of the adipogenic transcription factors PPARγ and C/EBPα [27].

In the present study, we demonstrated that high phosphorus can induce fully differentiated mature adipocytes into osteoblast-like cells with increased expression of Runx2 and decreased expression of PPARγ and CEBP, leading to subsequent mineralization. Interestingly, the cells maintained expression of PPARγ and CEBP and still had lipid filled cytoplasm characteristic of adipocytes (Fig. 1). This indicated that hyperphosphatemia either converts only some cells, or that it is the ratio of the various transcription factors that regulates calcification. We also found a parallel increase in osteopontin, an inhibitor of calcification secreted by osteoblasts and injured VSMC that has been identified in areas of calciphylaxis in human biopsies [3]. It is important to point out that not all patients with hyperphosphatemia develop calciphylaxis, nor do all patients with calciphylaxis have hyperphosphatemia, suggesting that other factors (for example oxidative stress or hypoxia) are also involved.

Adipose tissue is now recognized as a biologically active tissue involved in signaling through endocrine, paracrine and autocrine mechanisms. We demonstrated that the calcification of VSMC is significantly enhanced when co-cultured with adipocytes compared to VSMC alone. In contrast, VSMC had no effect on adipocyte calcification when co-cultured together. These results suggest that adipocytes have the potential to stimulate VSMC calcification and this effect is unidirectional. Adipocytes are now appreciated to serve as important mediators of inflammation and secrete several proteins such as leptin, adiponectin and VEGF-A [28,29]. Leptin treated animals have increased vascular calcification [30]. In vitro studies also demonstrate that leptin promotes osteoblast differentiation and mineralization of cultured vascular smooth muscle cells [31]. In the present study we found no direct effect of high phosphorus media on leptin or adiponectin secretion in adipocyte cultures, although leptin was markedly reduced in the co-cultures treated with sodium thiosulfate. Another paracrine factor is VEGF-A. Although produced by endothelial cells it is also secreted from human adipocytes and can induce VSMC proliferation [29]. VEGF-A also stimulates VSMC calcification in vitro [32]. Our results demonstrate that high phosphorus increased VEGF from adipocytes, and that this was partially reduced with sodium thiosulfate. Although the increase was modest, the change of VEGF-A secretion of 1 ng/ml from adipocytes observed in our study was shown to stimulate VSMC proliferation as reported by Schlich et al. [29].

Clinically sodium thiosulfate has been used to treat calciphylaxis with reduced mortality [8]. In uremic rats sodium thiosulfate inhibits calcification in the heart and kidneys [33] and prevents nephrolithiasis [34]. Initial studies suggested this was due to the physicochemical effect of increased solubility of calcium thiosulfate although direct studies on arterial rings did not confirm such a mechanism [35]. An alternative hypothesis is that sodium thiosulfate reduces oxidative stress [33,36]. The results from the current study clearly demonstrated that sodium thiosulfate directly inhibits high phosphorus-induced calcification in adipocytes but had no effect on VSMC calcification. Furthermore, sodium thiosulfate did prevent adipocyte enhancement of VSMC calcification. Sodium thiosulfate also decreased the secretion of VEGF and leptin from adipocytes. Translating these studies to humans, one can hypothesize that sodium thiosulfate may stop the adipocyte mediated VSMC calcification and thus prevent the formation of new lesions but may also halt progression of existing lesions in arterioles that are already damaged. In conclusion, adipocytes are capable of calcifying in media that contains high phosphorus by altering transcription factor expression and in a paracrine manner that may be mediated by VEGF or leptin. Sodium thiosulfate directly affects adipocyte calcification and prevents the ability of adipocytes to potentiate VSMC calcification.

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References

[1] S.M. Moe, N.X. Chen, Calciphylaxis and vascular calcification: a continuum of extra-skeletal osteogenesis, Pediatr. Nephrol. 18 (2003) 969–975.
[2] T. Coates, G.S. Kirkland, R.B. Dymock, B.F. Murphy, J.K. Brealey, T.H. Mathew, A.P. Disney, Cutaneous necrosis from calcific uremic arteriolopathy [see comments], Am. J. Kidney Dis. 32 (1998) 384–391.
[3] S. Ahmed, K.D. O'Neill, A.F. Hood, A.P. Evan, S.M. Moe, Calciphylaxis is associated with hyperphosphatemia and increased osteopontin expression by vascular smooth muscle cells, Am. J. Kidney Dis. 37 (2001) 1267–1276.
[4] A. Fine, J. Zacharias, Calciphylaxis is usually non-ulcerating: risk factors, outcome and therapy, Kidney Int. 61 (2002) 2210–2217.
[5] C. Saifan, M. Saad, E. El-Charabaty, S. El-Sayegh, Warfarin-induced calciphylaxis: a case report and review of literature, Int. J. Gen. Med. 6 (2013) 665–669.
[6] W.C. O'Neill, Treatment of vascular calcification, Kidney Int. 74 (2008) 1376–1378.
[7] G. Schlieper, V. Brandenburg, M. Ketteler, J. Floege, Sodium thiosulfate in the treatment of calcific uremic arteriolopathy, Nat. Rev. Nephrol. 5 (2009) 539–543.
[8] L. Noureddine, M. Landis, N. Patel, S.M. Moe, Efficacy of sodium thiosulfate for the treatment for calciphylaxis, Clin. Nephrol. 75 (2011) 485–490.
[9] S.M. Moe, K.D. O'Neill, D. Duan, S. Ahmed, N.X. Chen, S.B. Leapman, N. Fineberg, K. Kopecky, Medial artery calcification in ESRD patients is associated with deposition of bone matrix proteins, Kidney Int. 61 (2002) 638–647.
[10] N.X. Chen, K.D. O'Neill, D. Duan, S.M. Moe, Phosphorus and uremic serum up-regulate osteopontin expression in vascular smooth muscle cells, Kidney Int. 62 (2002) 1724–1731.
[11] H. Tamez, C. Zoccali, D. Packham, J. Wenger, I. Bhan, E. Appelbaum, Y. Pritchett, Y. Chang, R. Agarwal, C. Wanner, D. Lloyd-Jones, J. Cannata, B.T. Thompson, D. Andress, W. Zhang, B. Singh, D. Zehnder, A. Pachika, W.J. Manning, A. Shah, S.D. Solomon, R. Thadhani, Vitamin D reduces left atrial volume in patients with left ventricular hypertrophy and chronic kidney disease, Am. Heart J. 164 (2012). 902–909.e902.
[12] W.L. Lau, E.M. Leaf, M.C. Hu, M.M. Takeno, M. Kuro-o, O.W. Moe, C.M. Giachelli, Vitamin D receptor agonists increase klotho and osteopontin while decreasing aortic calcification in mice with chronic kidney disease fed a high phosphate diet, Kidney Int. 82 (2012) 1261–1270.
[13] N.X. Chen, F. Kircelli, K.D. O'Neill, X. Chen, S.M. Moe, Verapamil inhibits calcification and matrix vesicle activity of bovine vascular smooth muscle cells, Kidney Int. 77 (2010) 436–442.
[14] S.R. Contiguglia, A.C. Alfrey, N.L. Miller, D.E. Runnells, R.Z. Le Geros, Nature of soft tissue calcification in uremia, Kidney Int. 4 (1973) 229–235.

[15] G.A. Block, J.H. Ix, M. Ketteler, K.J. Martin, R.I. Thadhani, M. Tonelli, M. Wolf, H. Juppner, K. Hruska, D.C. Wheeler, Phosphate homeostasis in CKD: report of a scientific symposium sponsored by the National Kidney Foundation, Am. J. Kidney Dis. 62 (2013) 457–473.
[16] A.J. Bleyer, M. Choi, B. Igwemezie, E. de la Torre, W.L. White, A case control study of proximal calciphylaxis [see comments], Am. J. Kidney Dis. 32 (1998) 376–383.
[17] A.R. Mazhar, R.J. Johnson, D. Gillen, J.C. Stivelman, M.J. Ryan, C.L. Davis, C.O. Stehman-Breen, Risk factors and mortality associated with calciphylaxis in end-stage renal disease, Kidney Int. 60 (2001) 324–332.
[18] A. Fine, Relevance of C-reactive protein levels in peritoneal dialysis patients, Kidney Int. 61 (2002) 615–620.
[19] C.B. Raymond, L.D. Wazny, A.R. Sood, Sodium thiosulfate, bisphosphonates, and cinacalcet for calciphylaxis, CANNT J. 19 (2009) 25–27 (quiz 28–29).
[20] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143–147.
[21] P. Ducy, G. Karsenty, Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene, Mol. Cell. Biol. 15 (1995) 1858–1869.
[22] J. Kong, G.H. Kim, M. Wei, T. Sun, G. Li, S.Q. Liu, X. Li, I. Bhan, Q. Zhao, R. Thadhani, Y.C. Li, Therapeutic effects of vitamin D analogs on cardiac hypertrophy in spontaneously hypertensive rats, Am. J. Pathol. 177 (2010) 622–631.
[23] R. Przybylski, S. McCune, B. Hollis, R.U. Simpson, Vitamin D deficiency in the spontaneously hypertensive heart failure [SHHF] prone rat, Nutr. Metab. Cardiovasc. Dis. 20 (2010) 641–646.
[24] F. Mosna, L. Sensebe, M. Krampera, Human bone marrow and adipose tissue mesenchymal stem cells: a user's guide, Stem Cells Dev. 19 (2010) 1449–1470.
[25] J.H. Choi, Q. Ke, S. Bae, J.Y. Lee, Y.J. Kim, U.K. Kim, C. Arbeeny, R. Thadhani, P.M. Kang, Doxercalciferol, a pro-hormone of vitamin D, prevents the development of cardiac hypertrophy in rats, J. Card. Fail. 17 (2011) 1051–1058.
[26] J. Justesen, S.B. Pedersen, K. Stenderup, M. Kassem, Subcutaneous adipocytes can differentiate into bone-forming cells in vitro and in vivo, Tissue Eng. 10 (2004) 381–391.
[27] T. Takahashi, Overexpression of Runx2 and MKP-1 stimulates transdifferentiation of 3T3-L1 preadipocytes into bone-forming osteoblasts in vitro, Calcif. Tissue Int. 88 (2011) 336–347.
[28] S. Galic, J.S. Oakhill, G.R. Steinberg, Adipose tissue as an endocrine organ, Mol. Cell. Endocrinol. 316 (2010) 129–139.
[29] R. Schlich, M. Willems, S. Greulich, F. Ruppe, W.T. Knoefel, D.M. Ouwens, B. Maxhera, A. Lichtenberg, J. Eckel, H. Sell, VEGF in the crosstalk between human adipocytes and smooth muscle cells: depot-specific release from visceral and perivascular adipose tissue, Mediators Inflamm. 2013 (2013) 982458.
[30] M. Zeadin, M. Butcher, G. Werstuck, M. Khan, C.K. Yee, S.G. Shaughnessy, Effect of leptin on vascular calcification in apolipoprotein E-deficient mice, Arterioscler. Thromb. Vasc. Biol. 29 (2009) 2069–2075.
[31] M.G. Zeadin, M.K. Butcher, S.G. Shaughnessy, G.H. Werstuck, Leptin promotes osteoblast differentiation and mineralization of primary cultures of vascular smooth muscle cells by inhibiting glycogen synthase kinase (GSK)-3beta, Biochem. Biophys. Res. Commun. 425 (2012) 924–930.
[32] L. Mikhaylova, J. Malmquist, M. Nurminskaya, Regulation of in vitro vascular calcification by BMP4, VEGF and Wnt3a, Calcif. Tissue Int. 81 (2007) 372–381.
[33] A. Pasch, T. Schaffner, U. Huynh-Do, B.M. Frey, F.J. Frey, S. Farese, Sodium thiosulfate prevents vascular calcifications in uremic rats, Kidney Int. 74 (2008) 1444–1453.
[34] J.R. Asplin, S.E. Donahue, C. Lindeman, A. Michalenka, K.L. Strutz, D.A. Bushinsky, Thiosulfate reduces calcium phosphate nephrolithiasis, J. Am. Soc. Nephrol. 20 (2009) 1246–1253.
[35] W.C. O'Neill, K.I. Hardcastle, The chemistry of thiosulfate and vascular calcification, Nephrol. Dial. Transplant. 27 (2012) 521–526.
[36] H. Yatzidis, Successful sodium thiosulphate treatment for recurrent calcium urolithiasis, Clin. Nephrol. 23 (1985) 63–67.