Ascorbic acid regulates osterix expression in osteoblasts by activation of prolyl hydroxylase and ubiquitination-mediated proteosomal degradation pathway

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Xing W, Pourteymoor S, Mohan S. Ascorbic acid regulates osterix expression in osteoblasts by activation of prolyl hydroxylase and ubiquitination-mediated proteosomal degradation pathway. Physiol Genomics 43: 749–757, 2011. First published April 5, 2011; doi:10.1152/physiolgenomics.00229.2010.—Mouse genetic studies reveal that ascorbic acid (AA) is essential for osteoblast (OB) differentiation and that osterix (Osx) was a key downstream target of AA action in OBs. To determine the molecular pathways for AA regulation of Osx expression, we evaluated if AA regulates Osx expression by regulating production and/or actions of local growth factors and extracellular matrix (ECM) proteins. Inhibition of actions of IGFs by inhibitory IGFBP-4, BMPs by noggin, and ECM-mediated integrin signaling by RGD did not block AA effects on Osx expression in OBs. Furthermore, blockade of components of MAPK signaling pathway had no effect on AA-induced Osx expression. Because AA is required for prolyl hydroxylase domain (PHD) activity and because PHD-induced prolyl-hydroxylation targets proteins to proteosomal degradation, we next tested if AA effect on Osx expression involves activation of PHD to hydroxylate and induce ubiquitin-proteosome-mediated degradation of transcriptional repressor(s) of Osx gene. Treatment of OBs with dimethylloxal glycine and ethyl 3, 4-dihydroxybenzoate, known inhibitors of PHD, completely blocked AA effect on Osx expression and OB differentiation. Knockdown of PHD2 expression by Lentivirus-mediated shRNA abolished AA-induced Osx induction and alkaline phosphatase activity. Furthermore, treatment of OBs with MG115, inhibitor of proteosomal degradation, completely blocked AA effects on Osx expression. Based on these data, we conclude that AA effect on Osx expression is mediated via a novel mechanism that involves PHD2 and proteosomal degradation of a yet to be identified transcriptional repressor that is independent of BMP, IGF-I, or integrin-mediated signaling in mouse OBs.

IN PREVIOUS STUDIES, WE and others found that mice with deletion of the gulonolactone oxidase gene (GULO), which is involved in the synthesis of an antioxidant ascorbic acid (AA), was responsible for AA deficiency and impairment of differentiated functions of osteoblast, bone fracture, and premature death in spontaneous fracture (sfx) mice (16, 32). Treatment of the mutant mice with AA in drinking water completely rescued the bone phenotypes in vivo and prevented them from premature death (32). Similarly, epidemiological studies have provided evidence for the increased risk of bone fractures caused by decreased bone formation in patients with vitamin C deficiency (26, 29, 67). In terms of mechanism for reduced bone formation during vitamin C deficiency, we have earlier reported that the impaired differentiation of bone marrow stromal (BMS) cells derived from sfx mice into osteoblasts in vitro can be rescued completely by treatment with a long acting vitamin C derivative, AA-2-phosphate (32). In subsequent studies, we found that the increase in osterix expression during in vitro differentiation of BMS cells is dependent on vitamin C (64). Furthermore, we found that AA induction of osterix mRNA expression occurred as early as 4 h after treatment, independent of new protein synthesis and mRNA stability (64). These studies strongly suggest a transcriptional regulation of osterix gene expression by AA. However, the molecular mechanism for AA regulation of osterix gene is unknown.

In terms of molecular pathways for the regulation of osteoblast differentiation, it is known that locally produced growth factors are important regulators (13, 41, 44, 45, 66). The actions of growth factors on osteoblasts are known to be mediated via activation of mitogen-activated protein kinase (MAPK) and the stress-activated protein kinase/c-Jun NH2-terminal kinase (JNK) signaling pathways (11, 17, 59). In addition, growth factors can interact with integrin signaling to regulate osteoblast functions (60). Thus, it is possible that vitamin C effects on osterix expression are mediated via vitamin C regulation of growth factor signaling pathways (59).

Vitamin C is an important cofactor for prolyl hydroxylase domain (PHD) proteins (28), which are members of the 2-oxoglutarate/iron-dependent dioxygenase superfamily (12). In mammals, PHD enzymes include PHD1, PHD2, and PHD3 (7). Both PHD1 and PHD2 contain more than 400 amino acid residues, while PHD3 has fewer than 250. All three members, however, contain the highly conserved hydroxylase domain in the catalytic carboxy-terminal region. The importance of AA for hydroxylation and secretion of procollagen to form stable triple-helical collagen both in the growing and in the mature connective tissue is well established (24, 37). The potential mechanisms by which AA directs the differentiation of multipotent progenitor cells towards bone cells are believed to be mediated through collagen matrix syntheses, cell-matrix interaction, and activation of integrin signaling (10, 14). However, the GULO knockout mice have normal levels of mature collagen production in the tail, mammary gland, and tumors (32, 36). In addition, it has been demonstrated that AA can stimulate the expression of a number of osteogenic marker genes in the presence of collagen synthesis inhibitors and can induce chondrocyte hypertrophy independent of production of a collagen-rich matrix (48). These studies indicate that additional
mechanisms besides the collagen-mediated signaling may be involved in mediating AA effects on osteoblast differentiation.

Recent studies have found that PHDs are negative regulators of the hypoxia-inducible factor (HIF)α (3, 22). The hydroxylation of specific proline residues (Pro-402 and Pro-564) in the COOH-terminal oxygen-dependent degradation domains of the HIF1α by PHDs, primarily the PHD2 isoform, leads to the targeting of HIF1α for ubiquitination through an E3 ligase complex initiated by the binding of the von Hippel-Lindau protein (pVHL) and subsequent proteasomal degradation (3, 22). Hydroxylation of HIF1α requires molecular oxygen and iron. Under the hypoxia condition, PHDs are inhibited, and the HIF1α accumulates in the cytoplasm, which it translocates to the nucleus and binds to DNA to regulate hypoxia-responsive genes including VEGF, Runx2, and osterix (15, 55). Besides the HIF1α pathway underlying regulation of angiogenesis and osteogenesis during skeletal development, as demonstrated in other reports, PHD can hydroxylate other substrates including IKK-β, β2-adrenergic receptor, HIF1α-binding protein suppressor of cytokine signaling, and Argonaute and can influence their functions in a number of ways (6, 8, 39, 62). Because collagen prolyl hydroxylase is well known for its involvement in scurvy, in which ascorbate deficiency inhibits the enzyme resulting in defective collagen formation, we evaluated the feasibility that AA modulation of osterix gene expression is mediated via AA effects on PHD activity in osteoblasts.

MATERIALS AND METHODS

Antibodies and biological reagents. Antibody against β-actin was purchased from Sigma. Antibody specific to mouse osterix was from Abcam (Cambridge, MA). Antibody against HIF1α was a product of Novus Biologicals (Littleton, CO). Antibody specific to PHD2 was from Cell Signaling Technology (Boston, MA). The antibody has been used and published previously (35). Dimethylxylglyline (DMOG) was purchased from Cayman Chemical (Ann Arbor, MI). Peptides of MG115 (Z-Leu-Leu-Nva-H) and cyclic Arg-Gly-Asp (RGD) were from Peptides International (Louisville, KY) and Bachem (Torrance, CA), respectively. Recombinant mouse noggin was from R & D Systems (Minneapolis, MN). Kinase inhibitors of SB-203580, PD-98059, and SP-600125 were purchased from Calbiochem (San Diego, CA). Desferrioxamine (DFO), protocatechuic acid ethyl ester, aka ethyl 3, 4-dihydroxybenzoate (EDHB), and other chemicals were from Sigma. Recombinant human insulin-like growth factor binding protein-4 (BP-4) was purified from Escherichia coli (San Diego, CA). Desferrioxamine (DFO), protocatechuic acid ethyl ester, aka ethyl 3, 4-dihydroxybenzoate (EDHB), and other chemicals were from Sigma. Recombinant human insulin-like growth factor binding protein-4 (BP-4) was purified from Escherichia coli (San Diego, CA). Desferrioxamine (DFO), protocatechuic acid ethyl ester, aka ethyl 3, 4-dihydroxybenzoate (EDHB), and other chemicals were from Sigma. Recombinant human insulin-like growth factor binding protein-4 (BP-4) was purified from Escherichia coli (San Diego, CA). Desferrioxamine (DFO), protocatechuic acid ethyl ester, aka ethyl 3, 4-dihydroxybenzoate (EDHB), and other chemicals were from Sigma. Recombinant human insulin-like growth factor binding protein-4 (BP-4) was purified from Escherichia coli (San Diego, CA). Desferrioxamine (DFO), protocatechuic acid ethyl ester, aka ethyl 3, 4-dihydroxybenzoate (EDHB), and other chemicals were from Sigma. Recombinant human insulin-like growth factor binding protein-4 (BP-4) was purified from Escherichia coli (San Diego, CA). Desferrioxamine (DFO), protocatechuic acid ethyl ester, aka ethyl 3, 4-dihydroxybenzoate (EDHB), and other chemicals were from Sigma.
and/or BMP signaling is involved in regulating osterix expression, we examined the effects of pretreatment of MC3T3 cells with inhibitory BP-4 and noggin that will bind free IGF-I and BMP, respectively, on osterix expression. MC3T3-E1 cells were pretreated with 300 ng/ml of BP-4 and 250 ng/ml of noggin, respectively, for 30 min prior to treatment with AA or without AA. RNA was extracted for real-time RT-PCR 24 h after treatment. We found that neither BP-4 nor noggin pretreatment blocked AA-induced increase in osterix expression (Fig. 2, A and B). Exogenously added BP-4 alone blocked both basal and IGF-I-induced MC3T3-E1 proliferation under the culture conditions used in this study (data not shown). Furthermore, noggin alone blocked 10 ng/ml BMP-2-induced osterix expression by 52% (P < 0.01). These positive control data suggest that BP-4 and noggin at doses used were effective in blocking IGF and BMP actions, respectively. AA is known to induce collagen expression, which activates integrin signaling. Many members of the integrin family, including α5β1, α8β1, α1ββ3, αVβ3, αVβ5, αVβ6, and αVβ8, recognize an RGD motif within their ligands, including fibronectin, fibrinogen, vitronectin, von Willebrand factor, and many other large glycoproteins. Exogenous addition of excess RGD pep-

**RESULTS**

AA induces osterix expression in osteoblasts. To determine if the impairment in bone formation observed in our previous studies in sfx mice is attributable to decreased expression of osteoblast-specific transcription factors, we examined the effects of AA on the expression levels of various transcription factors that are known to be involved in osteoblast differentiation. We cultured primary calvarial osteoblasts derived from 4-day to 2-wk-old mice and MC3T3-E1 cells in AA-free α-MEM supplemented with or without AA for 24 h and evaluated the expression levels of osterix, Runx2, Dlx3, Dlx5, Msx1, and Msx2 by real-time PCR (Fig. 1A). We found that 24 h AA treatment stimulated osterix expression by 10-fold in both primary calvarial osteoblasts and MC3T3-E1 cells. In contrast to osterix, AA treatment did not cause significant changes in the expression levels of Runx2, Dlx3, Dlx5, and Msx2 in both primary calvarial osteoblasts and MC3T3-E1 cells. Msx1 expression was below detectable level in both of these cell types (data not shown). To confirm real-time PCR data, we treated the primary osteoblasts with AA for 72 h and examined osterix protein levels by Western blot (Fig. 1B). Consistent with the mRNA data, osterix protein levels in total cell extract were increased by threefold in AA-treated primary osteoblasts compared with the cellular osterix levels from the control cells without AA treatment (Fig. 1C). In contrast, Runx2 protein levels were not increased in AA-treated cultures.

AA induction of osterix expression is independent of IGF-I, bone morphogenetic protein, integrin, p38, and MAP/ERK kinase pathways. It has been known that growth factors such as bone morphogenetic protein (BMP) and IGF-1 positively regulate osterix expression in osteoblasts. Therefore, it is possible that AA regulates osterix expression through modulation of local growth factor actions or there is a cross talk between AA and growth factor signaling pathways. To determine if IGF-I...
It has been known that AA is an important osterix expression in the presence of JNK inhibitor. MEK inhibitors. However, AA treatment failed to stimulate absence of AA. Interestingly, AA-induced increase in osterix SP-600125 significantly increased osterix expression in the absence of AA. (Fig. 2) MAPK or MEK inhibitor did not significantly influence osterix expression compared with DMSO control in the absence of AA. Treatment of MC3T3-E1 cell proliferation. RGD peptide (5 μM) blocked 1% serum-induced MC3T3-E1 proliferation by 84% (P < 0.01), thus suggesting the inhibitor was biologically active.

The effects of growth factors on osteoblast differentiation and osterix expression are known to be mediated in part via activation of MAPK signaling pathway. To identify the signaling pathways by which AA regulates osterix expression, we pretreated MC3T3-E1 cells with DMSO or effective concentrations of inhibitors of p38 (200 nM SB-203580), MAP/ERK kinase (MEK, 10 μM U-0126), and JNK (200 nM SP-600125) for 30 min prior to treatment with AA or vehicle control. We found that treatment of MC3T3-E1 osteoblasts with p38 MAPK or MEK inhibitor did not significantly influence osterix expression compared with DMSO control in the absence of AA (Fig. 2D). However, treatment of the cells with JNK inhibitor SP-600125 significantly increased osterix expression in the absence of AA. Interestingly, AA-induced increase in osterix expression was not significantly affected by p38 MAPK or MEK inhibitors. However, AA treatment failed to stimulate osterix expression in the presence of JNK inhibitor.

AA stimulates osterix expression via modulating PHD enzyme activity. It has been known that AA is an important cofactor for PHD enzymes that catalyze posttranslational modifications of collagen and AA deficiency results in collagen maturation defects. PHDs also require Fe²⁺ for their enzymatic activity, and supplementation of the cells with FeCl₂ increases PHD activity, while treatment of the cells with DFO, an iron chelator, inhibits PHD’s ability to hydroxylate target proteins. To determine if AA effects on osterix expression are dependent on AA activation of PHD activity, we treated primary osteoblasts with DFO, FeCl₂, or vehicle control in the presence or absence of AA for 24 h and examined the consequence of modulation of PHD activity by Fe²⁺ on AA-induced osterix expression. We found that treatment of osteoblasts with DFO resulted in a 60% reduction in AA-induced osterix expression, while treatment of the cells with FeCl₂ caused a slight but insignificant increase in osterix expression (Fig. 3A). To further confirm that the AA-induced increase in osterix is mediated via a PHD-dependent mechanism, we treated primary osteoblasts with DMOG (500 μM) or EDHB (500 μM), known inhibitors of PHD, for 30 min prior to 24 h treatment with or without AA and examined the osterix mRNA and protein changes. We found that pretreatment with DMOG and EDHB completely blocked AA-induced increase in osterix expression in primary osteoblasts at both mRNA and protein levels (Fig. 3, B and C). Similar effects were seen in primary cultures of mouse calvarial osteoblasts (data not shown). As expected, the same DMOG treatment increased HIF1α protein levels, a well-known substrate of PHDs (Fig. 3D). Hydroxylation of two conserved proline residues in HIF1α by PHDs allows recognition and binding by the pVHL, ultimately resulting in polyubiquitination and proteosomal degradation of HIF1α. Consistent with the elevated HIF1α protein, the expression of VEGF, a known HIF1α target gene, was significantly increased in the cells treated with DMOG in the presence or absence of AA, whereas the AA treatment caused a reduction in VEGF expression in the absence of DMOG (Fig. 3E).

To determine which isoforms of PHDs are expressed in bone cells, we examined the transcripts of PHD1, PHD2, and PHD3 in primary calvarial osteoblasts and MC3T3-E1 cells by real-time RT-PCR. As shown in Fig. 4A, PHD2 was expressed at higher level than PHD1, and AA treatment significantly inhibited PHD2 but not PHD1 expression. The expression of PHD3 in bone cells was undetectable (data not shown). To evaluate the role of PHD2 in regulating AA induction of osterix expression, we knocked down PHD2 expression by lentivirus-mediated shRNA and examined osterix expression in response to AA treatment in MC3T3-E1 cells. We found that PHD2 expression was reduced by nearly 80% at both protein level and transcript level in the cells infected with mouse PHD2 shRNA compared with the cells transduced with scramble control shRNA (Fig. 4, B and C). PHD1 expression was not significantly different in control shRNA vs. PHD2 shRNA-
As expected, the mRNA expression of osterix was increased by 2.5-fold upon AA treatment in MC3T3-E1 cells expressing scramble control shRNA. However, AA-induced osterix induction was almost abolished in the cells expressing PHD2 shRNA (Fig. 4D). Consistent with impaired expression of osterix, AA-induced ALP activity was reduced by 66% in the MC3T3 cells expressing shRNA against PHD2 compared with the cells expressing control shRNA (Fig. 4E).

To further study the function of PHD2 on AA-mediated osteoblast differentiation, we treated primary calvarial osteoblasts with DMOG and vehicle control in the presence or absence of AA for 6 days. We found that treatment of AA increased ALP-stained area by more than threefold activity compared with the cells without AA treatment (Fig. 5A). However, addition of DMOG in the same AA-containing differentiation medium abolished AA-induced ALP expression as well as basal level of ALP expression (Fig. 5B).

Ubiquitination-mediated proteosomal degradation pathway is involved in mediating AA-induced increase in osterix expression. Posttranslational hydroxylation of proline residues of target proteins by PHDs lead to rapid decay via ubiquitin-proteosomal pathway. To determine if AA-induced osterix expression is mediated via a mechanism that involves prolyl hydroxylation and subsequent degradation of transcriptional suppressors or negative regulator of osterix transcription via ubiquitin-mediated pathway, we evaluated the consequence of inhibition of proteosomal degradation on osterix expression. Primary osteoblasts and MC3T3-E1 cells were treated with...
MG115 (25 μM) for 30 min prior to treatment with or without AA. We found that treatment of MG115 completely blocked AA-induced increase in osterix expression in both MC3T3-E1 osteoblasts and primary mouse calvarial osteoblasts (Fig. 6A). As expected, the same treatment of MC3T3 cells with MG115 increased VEGF expression as a consequence of MG115 blockade of HIF1α degradation (Fig. 6B).

**DISCUSSION**

Of the various transcription factors that regulate osteoblast differentiation, Runx2 and osterix have been widely accepted as master osteogenic factors since neither Runx2 nor osterix null mice form mature osteoblasts (20, 33). While osterix is known to act downstream of Runx2 and is regulated by growth factors (27, 33, 34), little is known on other key physiological regulators of osterix expression during bone development. In our previous studies, we have found that vitamin C is an important regulator of osterix expression during osteoblast differentiation, since AA is required for increased osterix expression during osteoblast differentiation (64) and since gulonolactone oxidase-deficient mice that were unable to synthesize AA develop spontaneous fractures at a very young age as a result of defective osteoblast maturation (32). Because of the established importance of AA in the regulation of osterix expression and because osterix and AA are critical regulators of osteoblast maturation, our goal in this study was to identify the molecular pathways for AA regulation of osterix expression.

In terms of mechanism for vitamin C regulation of osteoblast expression, it has been shown that AA regulates production of growth factors such as HGF and IGF-I (21, 58), which are known to regulate osterix expression (5, 47). Because conditional disruption of IGF-I in osteoblasts impaired osteoblast maturation and bone formation (13), we determined if AA effects on osterix expression is mediated via regulation of IGF actions. We found that blockade of IGF actions by inhibitory IGFBP-4 did not influence AA-induction of osterix expression in osteoblasts, thus suggesting that AA effect on osterix expression is not dependent on IGF action. Besides IGFs, the members of TGFβ/BMP superfamily also play critical roles in the regulation of osteoblast maturation and osterix expression (5, 27, 53). We, therefore, determined if inhibition of action of endogenously produced BMPs via exogenous addition of noggin modulated vitamin C effect on osterix expression. While noggin blocked the effect of exogenously added BMPs, it had no significant effect on AA-induced osterix expression, thus suggesting that AA effect on osterix expression is not dependent on BMP signaling pathway under the culture conditions used in this study.

Because AA has been shown to stimulate production of type I collagen in osteoblasts and because the interaction between α2-integrin and collagen has been shown to be involved in the activation of Runx2 and induction of osteoblast-specific gene expression (10, 61), we considered the possibility that AA induction of osterix expression may be mediated via activation of integrin signaling. In this study, we found that inhibition of integrin signaling via exogenous addition of echistatin did not block AA stimulation of osterix expression. Accordingly, treatment of osteoblasts with cyclic RGD peptide did not inhibit vitamin C effects on osterix expression, thus suggesting that type I collagen-mediated activation of integrin signaling is not a critical player in mediating AA effects on osterix gene transcription.

It is believed that IGF-I-mediated osterix expression requires activation of all three MAPK components (Erk1/2, p38, and JNK), whereas BMP-2 requires p38 and JNK signaling (5). Integrins are also known to activate MAPK, p38, and focal adhesion kinase to induce expression of osteoblast-specific transcription factors (43, 60, 61). Consistent with the results from inhibition of IGF-I, BMP, and integrin signaling, we found that pretreatment of MC3T3-E1 cells with effective concentrations of p38 MAPK and ERK1/2-specific inhibitors had no effect on AA-induced osterix expression. In contrast, BMP-2-induced osterix expression was completely blocked by p38 MAPK and JNK inhibitors (data not shown). Thus, our findings argue against a key role of endogenously produced IGF-I, BMP, and type I collagen in mediating vitamin C effects on osterix expression under culture conditions used in this study.

Vitamin C is an important cofactor of collagen prolyl hydroxylase, and several distinct prolyl hydroxylases are known to hydroxylate HIF transcription factors. In present study, we examined the effect of two well-studied chemical inhibitors of PHDs, DMOG and EHDB, on osterix expression. Both inhibitors completely abolished AA-induction of osterix expression in MC3T3-E1 cells as well as in primary osteoblasts. Besides vitamin C, iron and oxoglutarate are important cofactors for PHD activity. Accordingly, we found that treatment of MC3T3-E1 cells with DFO, which binds to iron, also inhibited AA induction of osterix expression. These findings provide first evidence that vitamin C effect on osterix expression is mediated via PHD-dependent mechanism.

Three isofoms of PHDs are widely distributed among different organs at the transcript level. PHD1 is expressed at the highest level in testes, whereas PHD3 is the highest in the heart (25). At protein level, however, PHD2 is the most abundant in all mouse organs examined (50). Consistent with previous studies, we found that PHD2 was predominantly expressed in

**Fig. 6. Effects of MG115, proteosomal degradation inhibitor, on AA-induced Osx and VEGF expression.** Primary osteoblasts and MC3T3-E1 osteoblasts were treated with vehicle or MG115 (25 μM) for 30 min prior to treatment with or without AA. RNA was extracted for real time RT-PCR 24 h after treatment. A: expression levels of osterix in primary osteoblasts and MC3T3-E1 cells, detected by real-time PCR. The results are expressed as fold change compared with the expression level of vehicle control in the same cells without AA treatment. *Statistical significance compared with expression level of control cells without AA treatment (P < 0.01, n = 3). B: expression levels of VEGF in primary osteoblasts, detected by real-time PCR. The results are expressed as fold change compared with the expression level of vehicle control in the same cells without AA treatment. *Statistical significance compared with expression level of control cells without AA treatment (P < 0.01, n = 3).
asparagine residues of their substrates such as HIF mRNA to osterix induction in osteoblasts. Knock-down of 80% PHD2 expression by lentivirus-mediated shRNA abolished AA-induced osterix induction in osteoblasts. Interestingly, PHD2 has been reported to be a major regulator of transcription factor HIF1α (2), and knock-out of PHD2 causes embryonic lethality during organogenesis with abnormal placental and cardiac morphology (52), while conditional knockout of PHD2 in somatic cells displayed reduced body size, increased angiogenesis, and premature death (31). Mice with conditional deletion of PHD2 in chondrocytes were born normal but quickly became growth-retarded because of increased cartilage matrix mineralization (23). In contrast, knockout of PHD1 in mice exhibited no apparent abnormalities in cardiovascular, hematopoietic, or placental morphology and skeleton (51, 52). PHD1/PHD3 double deficiency led to hepatic accumulation of HIF2α, but not HIF1α (50). Our data together with the phenotypes of specific PHD gene knockout data strongly indicate that PHD2 may play an important role in mediating AA effects on osterix expression and osteoblast differentiation, and PHD1, although expressed, cannot compensate for the loss of PHD2 in mediating osterix induction in osteoblasts.

PHD enzymes catalyze hydroxylation of the specific proline and asparagine residues of their substrates such as HIF1α (9). Prolyl-4 hydroxylation at two sites within a central degradation domain of HIF1α by PHDs, mainly PHD2, mediates interactions with the VHL E3 ubiquitin ligase complex that targets HIF1α for proteosomal degradation (4, 7). Hydroxylation of an asparaginyl residue in the COOH-terminal activation domain by PHDs inhibits transcriptional activity by preventing interaction with co-activator, p300/CREB (30). The HIF hydroxylases are dependent on ascorbate, and activation of PHDs in AA-treated cells allows HIF1α to undergo proteolysis and becomes transcriptionally inactive, therefore inhibiting its target gene expression. Accordingly, we found that inhibition of PHDs by DMOG increased HIF1α protein levels in osteoblasts. Furthermore, treatment of osteoblasts with DMOG increased HIF1α target gene, VEGF expression. In recent studies, it has been shown that HIF1α is a transcriptional activator of osterix gene transcription via hypoxia response element present in osterix gene promoter (55). If the vitamin C effect on osterix is mediated via HIF1α-dependent mechanism, we would then anticipate increased osterix expression in cultures treated with PHD inhibitors. However, we found that treatment of osteoblasts with DMOG and EDHB increased HIF1α level but decreased osterix expression and osteoblast differentiation. Furthermore, conditional disruption of HIF1α in cells of osteoblastic lineage impaired skeletal development (38, 54). In this regard, mice with conditional disruption of HIF1α in the condensing mesenchyme had shortened bones, less-mineralized skulls, and widened sutures due to massive apoptosis and altered proliferation of chondrocytes in growth plate (38). Because previous studies have demonstrated that HIF1α is a positive regulator of bone formation, it is unlikely that HIF1α is a repressor that mediates vitamin C effects on osterix expression.

Because prolyl hydroxylation of target proteins by PHDs leads to ubiquitin-mediated proteosomal degradation and because pretreatment of MC3T3-E1 cells with inhibitors of proteosomal degradation blocked AA-induced osterix expression, we speculate that AA-mediated activation of PHDs leads to prolyl hydroxylation and subsequent degradation of one or more negative regulators of osterix transcription. We ruled out Runx2 as a potential candidate although it is upstream of osterix and dispensable for osterix expression for two reasons: First, Runx2 is a positive regulator of osterix expression. Second, AA treatment did not increase protein levels of Runx2.

In recent studies, tumor suppressor gene, p53, has been identified as a negative regulator of osterix expression (56). It was found that p53 knock-out mice exhibited increased bone formation and osteoblast differentiation that could be explained by increased osterix expression. Furthermore, osteoblast could be repressed by p53 in reporter assays. Stat1 and E4BP4 have also been identified as negative regulators of osterix expression (46, 49). PHDs also regulate the stability of IKK, therefore affecting NF-κB pathway (65). We recently published that NFE2L1 could in part mediate AA effects on osterix expression in vitro and in vivo. Future studies will address whether NFE2L1, NF-κB, p53, and/or other suppressors of osterix expression are direct or indirect targets of PHD2 to mediate AA action in osteoblasts.

In summary, we have provided first experimental data for involvement of PHD-dependent mechanism for mediating the effects of vitamin C on osterix expression and osteoblast differentiation. Future identification of the PHD target transcriptional suppressor/s could lead to development of therapies to increase osteoblast differentiation and promote bone formation to treat metabolic bone disorders.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

15. Huang LE, Bunn HF. Hypoxia-inducible factor and its biomedical relevance. J Biol Chem 278: 19575–19578, 2003.
35. Standal T, Abildgaard N, Fagerli UM, Storbals D, Hjertner O, Borset M, Sundan A. HGF inhibits BMP-induced osteoblastogenesis: possible...


