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Prolyl-hydroxylase Inhibition and HIF Activation in Osteoblasts Promotes an Adipocytic Phenotype

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Abstract Bone is a dynamic environment where cells sense and adapt to changes in nutrient and oxygen availability. Conditions associated with hypoxia in bone are also associated with bone loss. In vitro hypoxia (2% oxygen) alters gene expression in osteoblasts and osteocytes and induces cellular changes including the upregulation of hypoxia inducible factor (HIF) levels. Our studies show that osteoblasts respond to hypoxia (2% oxygen) by enhancing expression of genes associated with adipocyte/lipogenesis phenotype (C/EBPβ, PPARγ2, and aP2) and by suppressing expression of genes associated with osteoblast differentiation (alkaline phosphatase, AP). Hypoxia increased HIF protein levels, hypoxic response element (HRE) binding, and HRE-reporter activity. We also demonstrate that prolyl-hydroxylases 2 and 3 (PHD2, PHD3), one of the major factors coordinating HIF degradation under normoxic but not hypoxic conditions, are induced in osteoblasts under hypoxic conditions. To further determine the contribution of PHDs and upregulated HIF activity in modulating osteoblast phenotype, we treated osteoblasts with a PHD inhibitor, dimethyloxaloylglycine (DMOG), and maintained cells under normoxic conditions. Similar to hypoxic conditions, HRE reporter activity was increased and adipogenic gene expression was increased while osteoblastic genes were suppressed. Taken together, our findings indicate a role for PHDs and HIFs in the regulation of osteoblast phenotype. J. Cell. Biochem. 100: 762–772, 2007.
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Key words: osteoblast; bone; hypoxia; adipocyte; PPARγ; prolylhydroxylase; differentiation; DMOG

Hypoxia is a demonstrated regulator of gene expression and cell function. Under hypoxic conditions, cells increase expression of genes associated with glycolysis (such as glyceraldehyde phosphate 3 dehydrogenase, GAPDH) and vascularization (vascular endothelial growth factor, VEGF) [Semenza, 2000; Seagroves et al., 2001]. This response allows cells to survive metabolically by meeting ATP demands through non-oxidative pathways until blood vessels are formed and normal oxygen levels are restored. Survival responses to hypoxia, while being beneficial for viability, can also modify or suppress cellular phenotype and function [Sahai et al., 1994; Matsuda et al., 1998; Desplat et al., 2002; Song et al., 2002; Jiang and Mendelson, 2003].

Bone is a dynamic environment that exhibits cell sensing, metabolic adaptation, and changes in oxygen availability [Kofoed, 1986; Kiaer et al., 1988, 1992; Otter et al., 1999; Harrison et al., 2002; Szymanski et al., 2002; Ionescu and Schoon, 2003]. Osteoblasts are one of the cell types in bone that are capable of responding to hypoxia. Previously, we and others have shown that runx2 and alkaline phosphatase (AP) expression (markers of osteoblast lineage selection and differentiation, respectively) are markedly suppressed in osteoblasts under hypoxic conditions [Park et al., 2002; Ontiveros et al., 2004; Salim et al., 2004]. These findings

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correspond with reports demonstrating that conditions of decreased bone oxygenation such as decreased arterial supply (chronic intramedullary pressure [Kiaer et al., 1992; Otter et al., 1999]) and inflammation (osteoarthritis [Kofoed, 1986; Kiaer et al., 1988]) are associated with bone loss. For example, ligation of bone nutrient arteries, lower extremity arterial disease and chronic pulmonary disease lead to decreased bone pO_2 and correspondingly bone loss in affected limbs [Kiaer et al., 1992; Vogt et al., 1997; Harrison et al., 2002; Szymanski et al., 2002; Ionescu and Schoon, 2003]. Associations between knee and hip inflammation, bone loss, and decreased bone pO_2 levels also exist [Kofoed, 1986; Kiaer et al., 1988].

Hypoxia is known to cause a variety of changes in cells including significant generation of reactive oxygen species [Carriere et al., 2004; Wang et al., 2005] and induction of hypoxia inducible factor (HIF) levels and transcriptional activity [Semenza, 1998, 2003]. Elevated protein levels and activity of HIFs, helix-loop-helix transcription factors, occurs in nearly all cells immediately upon hypoxic challenge. HIFs are the major regulators of a cell's responses to hypoxia, although there are some HIF-independent pathways [Yun et al., 2005]. Three major cytoplasmic HIFα subunits have been identified (1, 2, and 3) and all are active as dimers with one of two nuclear partners, HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator, ARNT) or ANRT2. Protein stability of HIFα subunits is highly regulated by oxygen tension and leads to HIFα accumulation under hypoxic conditions. Under normoxic conditions, HIF instability is incurred by hydroxylation of prolines 402 and 564 by proline hydroxylases (PHDs) 1, 2, and 3 [Ivan et al., 2001; Jaakkola et al., 2001]. The hydroxylated HIFα is recognized by von Hippel–Lindau protein (VHL [Zhu and Bunn, 2001]), and subsequently processed for ubiquitination and proteosome degradation [Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Yu et al., 2001]. Under hypoxic conditions, PHDs display a decreased activity, either due to a direct loss of the oxygen substrate or by an indirect mechanism involving mitochondrial formed reactive oxygen. HIFα translocates to the nucleus and is then capable of forming heterodimers with HIF-1β, which is constitutively expressed. The HIFα/HIFβ complex can then bind to hypoxia response elements (HREs) located in gene promoters to regulate transcription [Jiang et al., 1996; Semenza, 1998].

Targeted deletion of HIF-1α in the cartilaginous component of the developing growth plate suggests a critical role for HIFs in chondrocyte survival and maintenance of cell phenotype [Schipani et al., 2001]. HIFs are expressed in osteoblasts and osteocytes, and their protein levels are induced under hypoxic conditions [Akeno et al., 2001; Gross et al., 2001]. While it is clear that hypoxia and HIFs are involved in osteoblast VEGF expression [Steinbrech et al., 2000; Akeno et al., 2001; Kim et al., 2002], less is known about how hypoxia and associated HIF induction regulate osteoblast phenotype and function. Previously, we reported that hypoxia suppresses runx2 mRNA levels in osteoblasts [Ontiveros et al., 2004]. Here we examine HIF and PHD expression in response to hypoxia, and the role of PHD and HIF activity in suppressing markers of osteoblast maturation and enhancing adipocyte phenotype. Our findings suggest that PHD activity and its subsequent regulation of HIF stability are critical regulators of osteoblast phenotype.

MATERIALS AND METHODS

Osteoblast Cells

Studies employed mouse calvaria-derived non-transformed osteoblast cell line, MC3T3-E1 (passages 12–19), obtained from Riken Cell Bank, Japan, which differentiates and produces a mineralized matrix [Quarles et al., 1992] analogous to normal diploid rat osteoblasts [Lian and Stein, 1992] and bone development [Rodan et al., 1988]. Osteoblasts were plated at 20,000 cells/cm^2 surface area and fed every other day with alpha-MEM containing 10% fetal calf serum. After 4 days, the media were supplemented with 25 μg/ml ascorbic acid and 2 mM inorganic phosphate to promote osteoblast differentiation and bone formation. Cells were cultured under standard normoxic conditions for 14 days to promote differentiation. Lipid staining was performed by fixing osteoblasts in formalin for 20 min at room temperature and staining with Oil Red O for 40 min. Unincorporated stain was rinsed off with distilled water. Ethanol (100%) was added to cells for 15 min in order to remove the incorporated dye which was subsequently read at 508 nm [Stewart et al., 2004].

Hypoxic Experiments and Induction of HIF Activity

Osteoblasts were maintained under normoxic, osteoblast differentiating conditions for 14 days. On day 14, control dishes continued to be maintained in incubators containing 21% oxygen and 5% CO_2. For hypoxic conditions, dishes were moved into a hypoxic incubator where air in the incubator was replaced with nitrogen yielding 2% oxygen conditions. For time points taken at 24 h and greater, cells were fed 24 h prior to harvest (for a 48 h culture, cells were removed, and quickly fed and replaced; the latter step did not influence the response compared to not feeding). Cells were fed to assure that the medium contained a sufficient amount of glucose and full pH buffering capacity (these parameters were measured in all experiments). To examine HIF effects in the absence of hypoxia we also utilized an inhibitor of PHD activity, dimethyloxaloylglycine (DMOG; Cayman Chemicals). Cells were grown under normoxic conditions and were treated for 24 h with 5 mM DMOG, a concentration previously demonstrated by others to induce HIF levels in other cell types [Hanson et al., 2003].

RNA Analyses

RNA was extracted using the TRI Reagent RNA isolation reagent (Molecular Research Center, Inc., Cincinnati, OH). Integrity of the RNA was verified by formaldehyde-agarose gel electrophoresis. Two-step quantitative RT-PCR was performed to verify gene expression. First strand cDNA was synthesized by reverse transcription of 2 μg RNA, using the Superscript II Kit and oligo $dT_{(12-18)}$ primers (Invitrogen Life Technologies, Carlsbad, CA). cDNA (1 μl) was amplified by real time PCR, having a final volume of 25 μl, by using the Biorad SYBR Green Mix (Biorad, Hercules, CA). AP and Runx2, genes associated with osteoblast differentiation, were analyzed by using HPRT as the internal control as previously described [Vengellur and LaPres, 2004; Botolin et al., 2005]. VEGF (a hypoxia-inducible gene) and C/EBPβ, PPARγ2 and aP2 (markers of adipocyte phenotype) were also measured with primers previously described [Ontiveros et al., 2004; Phan et al., 2004; Botolin et al., 2005]. Additional primer sets include HIF-1α (5′-TGG CTC CCT ATA TCC CAA TG-3′ and 5′-GGT CTG CTG GAA CCC AGT AA-3′), HIF-2α (5′-GAG CAA GCC TTC CAA GAC AC-3′ and 5′-TTC GCA CTG ATG GTC TTG TC-3′) and PHD1 (5′-GGA ACC CAC ATG AGG TGA AG-3′ and 5′-AAC ACC TTT CTG TCC CGA TG-3′), PHD2 (5′-GAA GCT GGG CAA CTA CAG GA-3′ and 5′-CAT GTC ACG CAT CTT CCA TC-3′), and PHD3 (5′-AAG TTA CAC GGA GGG GTC CT-3′ and 5′-GGC TGG ACT TCA TGT GGA TT-3′). Real time PCR was carried out and analyzed for 40 cycles, using iCycler software (Biorad, Hecules, CA). Each cycle consisted of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. The slopes of amplification curves were similar between genes of interest and HPRT; serial dilutions of samples gave consistent results. Gel electrophoresis and melting curves verified the integrity of single PCR products.

Transient Transfection Studies

Osteoblasts were transfected using Lipofectamine (Invitrogen, Carlsbad, CA) 8 days after plating as previously described [Ontiveros and McCabe, 2003]. Cells were transfected with an 3X HRE-luciferase reporter plasmid [Hogenesch et al., 1998] and an SV40-beta-galactosidase reporter, the later of which was not modulated, did not interfere with hypoxic responsiveness and served as a control for transfection efficiency. Fourteen days after plating, cells remained in normoxia or were transferred to hypoxic conditions. Twenty-four hours later reporter activity was read using Promega luciferase or Clontech beta-galactosidase assay systems and a luminometer.

Protein Analyses/Western Blotting

Whole cell extracts were obtained by scraping, pelleting, and resuspending cells in lysis buffer: 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1%Triton-X100, and 10% glycerol. Protein concentrations were quantitated using Biorad DC protein detection. Whole cell extracts (50 μg per lane) were used for Western blot analyses as previously described [McCabe et al., 1996]. Antibodies specific for HIF-1α (NB100-105K4) and HIF-2α (NB-100-122) were obtained from Novus Biologicals (Littleton, CO). Actin levels were used as a loading control; actin antibody (I-19) was purchased from Santa Cruz Bioltechnology (Santa Cruz, CA).

Electrophoretic Mobility Shift Analyses

Nuclear extracts were obtained by hypotonic lysis as previously described [McCabe et al., 1996]. Nuclear extracts (4 μg) were incubated for 20 min at room temperature with 100,000 cpm ^{32}P-end labeled (T4 polynucleotide kinase, Invitrogen) HRE oligonucleotide (TGC ATA CGT GGG CTC CAA CAG). Samples were loaded onto a pre-run (1 h, 200 V) 5% polyacrylamide gel and run for 2 h at 200 V at 4°C. Gels were dried and exposed to film at −80°C.

Cell Viability

Cell viability was assessed by LIVE/DEAD Viability/Cytotoxicity Kit for animal cells according to manufacturer protocol (Molecular Probes, Eugene OR) and caspase-3 by ApoAlert Caspase Colormetric Assay Kit BD (Clontech, Mountain View, CA).

Statistical Analyses

All statistical analyses were performed using Microsoft Excel data analysis program for Student *t*-test analysis. Values are expressed as a mean ± SE, or relative to control values.

RESULTS

MC3T3-E1 pre-osteoblasts were grown under differentiating (media containing ascorbic acid and inorganic phosphate), normoxic conditions for 14 days. To test the influence of hypoxia on osteoblast differentiation, we exposed the day 14, differentiating osteoblasts to 21% (standard) versus 2% (hypoxia) oxygen conditions for 8, 24, 48, and 72 h. Levels of gene expression were compared to normoxic controls (21% oxygen) harvested at each corresponding time point and day post-plating. Values were expressed relative to HPRT levels, which were not modulated under hypoxic conditions, similar to previous reports [Vengellur and LaPres, 2004]. As expected, VEGF mRNA levels rapidly increased in 8 h of hypoxia exposure and continued to increase to levels approaching 30-fold greater, at 48 and 72 h, than those in osteoblasts under normoxic conditions (Fig. 1). AP mRNA levels showed a reciprocal relationship to VEGF, but equally dramatic. Specifically, after 8 h of hypoxic exposure AP mRNA levels decreased to less than 50% of normoxic control levels. By 48 and 72 h, the levels were 10% of normoxic controls (Fig. 1).

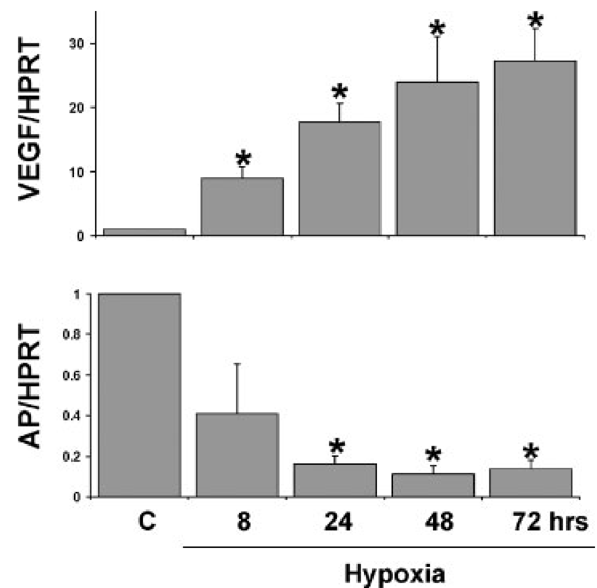

Fig. 1. Hypoxia increases vascular endothelial growth factor (VEGF) while decreasing alkaline phosphatase (AP) mRNA levels in MC3T3-E1 cells. Osteoblasts were grown for 14 days under standard normoxic conditions (21% O_2) and then were placed in hypoxia (2% O_2) for the times indicated. RNA was isolated, and VEGF and AP mRNA levels determined by RT-PCR. Levels of genes of interest were expressed relative to HPRT, a housekeeping gene that is not modulated by hypoxic conditions. Values represent averages of 4–10 separate experiments ± SE. $^*P \leq 0.05$.

Given the reciprocal nature between osteoblast versus adipocyte phenotypes, we examined the expression of adipocyte marker genes. Early markers of adipocyte phenotype, C/EBPβ and PPARγ2 expression, were increased two- to fivefold after 24 h of hypoxic exposure (Fig. 2). Significant induction of a later stage adipocyte marker, aP2, was evident within 8 h, increased further at 24 h (to roughly fivefold greater than control levels) and then further increased (to greater than 20-fold more than controls) after 72 h of hypoxia.

Examination of cell viability demonstrated no significant increase in osteoblast apoptosis or necrosis under conditions of hypoxia. Specifically, viability assays and caspase-3 activity measurements (at 24 and 48 h) were similar between control and hypoxia treated cells (data not shown). To prevent glucose deprivation (due to increase glycolysis), cells were fed prior to hypoxic treatment and/or 24 h prior to harvest. Media pH and glucose levels were monitored and remained within 0.2 units and 0.5 mM of controls, respectively.

Because of the critical role of HIFs in responsiveness to hypoxia, we examined HIF expression

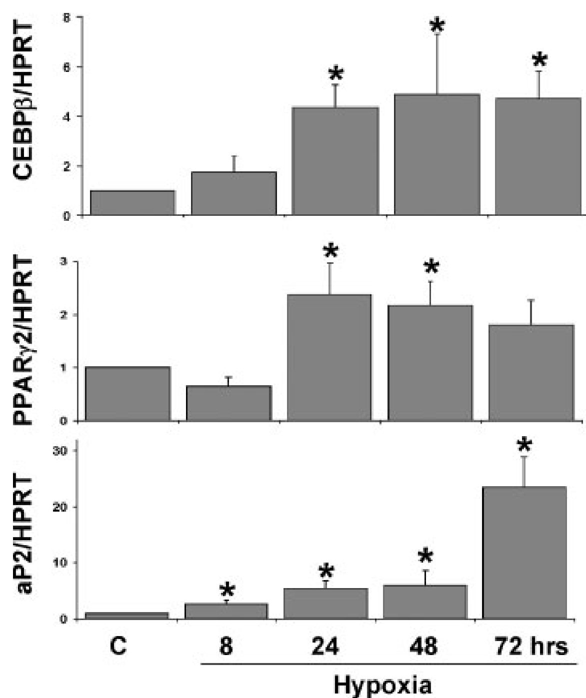

Fig. 2. Hypoxia increases mRNA levels of adipocytic marker genes in MC3T3-E1 cells. Osteoblasts were grown for 14 days under standard normoxic conditions (21% O_2) and then were placed in hypoxia (2% O_2) for the times indicated. RNA was isolated and C/EBPβ, PPARγ2, and aP2 mRNA levels determined by RT-PCR. Levels of genes of interest were expressed relative to HPRT, a housekeeping gene that is not modulated by hypoxic conditions. Values represent averages of 4–10 separate experiments ± SE. $^*P \leq 0.05$.

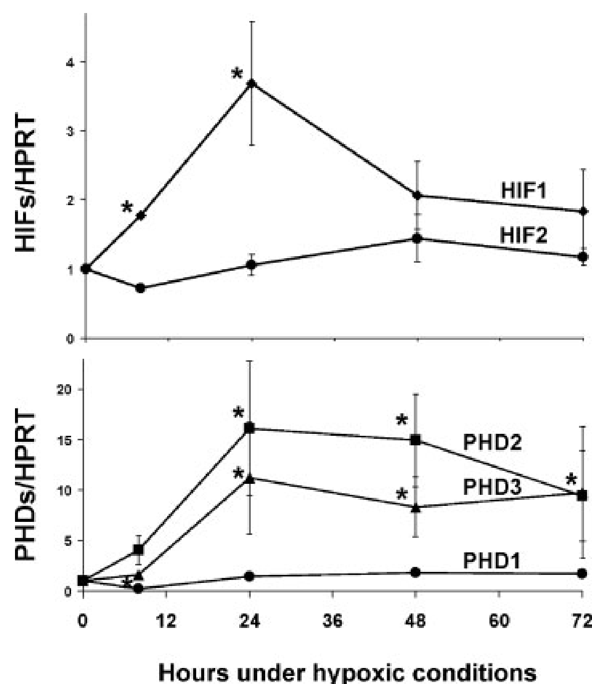

Fig. 3. PHD2, PHD3, and HIF-1α mRNA levels are induced by hypoxia. Osteoblasts were grown for 14 days under normoxic conditions (21% O_2) before being placed in hypoxia (2% O_2) for the times indicated. RNA levels of HIF and PHD members were measured by real time RT-PCR using primers that specifically recognize individual members. Levels of genes of interest were expressed relative to HPRT, a housekeeping gene that is not modulated by hypoxic conditions. Values represent averages of 4–10 separate experiments ± SE. $^*P \leq 0.05$ relative to normoxic conditions.

and the expression of one of the major regulators of HIF protein levels, prolyl-hydroxylases (PHDs). While HIF-2α mRNA levels were not modulated by hypoxia, HIF-1α mRNA levels were induced greater than threefold after a 24-h exposure to hypoxia (Fig. 3). By 48 h, HIF-1α mRNA levels returned to control, normoxic levels. Examination of the three PHD forms demonstrates that PHD2 and PHD3 mRNA levels were markedly induced to more than 10-fold greater than normoxic control levels.

Analysis of HRE DNA binding activity at 8, 24, and 48 h after the induction of hypoxia shows that HRE binding activity is increased at all time points (Fig. 4A). HRE binding in control, normoxic cells may represent some residual HIF activity as a result of non-hypoxic related factors such as stage of maturation or growth factor stimulation from the serum in the cell culture medium. Hypoxia induced protein levels of HIF-2α and also slightly increased HIF-1α levels (Fig. 4B) despite the induced expression of PHDs. To determine the functional influence of increased HIF levels, we also examined the activity of a transfected 3X HRE-reporter. Hypoxia dramatically induced HRE activity greater than 30-fold above normoxic control conditions after 24 h exposure (Fig. 4C).

To distinguish between HIF and non-HIF related effects, we next treated osteoblasts under normoxic conditions with a PHD inhibitor, DMOG. HRE-luciferase activity is induced by DMOG treatment (Fig. 5) to levels similar to those induced by hypoxia (30-fold greater than controls). Correspondingly, VEGF was induced and AP was suppressed, as seen under hypoxic conditions. In addition, C/EBPβ, PPARα2, and aP2 mRNA levels were all induced by a 24-h treatment with DMOG (Fig. 6). To confirm that these changes in gene expression can influence the phenotype the osteoblast cultures, cellular lipids were examined with oil red O staining. Measurements indicated that 24 h of hypoxia or DMOG treatment can increase cellular lipid

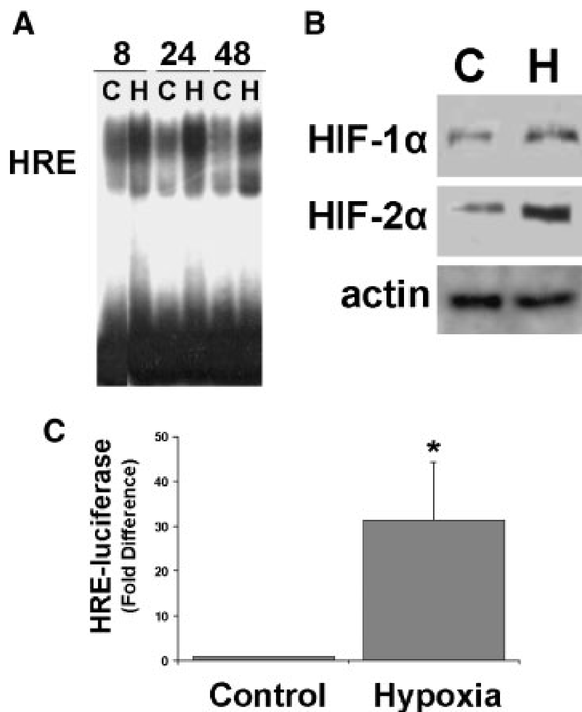

Fig. 4. HRE DNA binding, reporter activity, and HIF levels are increased in osteoblasts under hypoxic conditions. Osteoblasts were grown under normoxic conditions (21% O_2) for 14 days and placed into hypoxia (2% O_2) for the times indicated. **A**: Electrophoretic mobility shift assays were used to examine hypoxic response element (HRE) binding activity in nuclear extracts isolated from osteoblasts under control normoxic (C) or hypoxic (H) conditions. A representative autoradiograph is shown. **B**: Whole cell extracts obtained from osteoblasts under control normoxic (C) or hypoxic (H) conditions for 24 h were used for Western blot analyses with antibodies directed against HIF-1α, HIF-2α, or actin (a loading control). **C**: Osteoblasts were transfected with an HRE-luciferase reporter and placed in hypoxia for 24 h before assaying. Values represent HRE luciferase activity relative to SV40-betagalactosidase activity (to control for variations in transfection efficiency) and are expressed as averages of three separate experiments and relative to control levels ± SE.

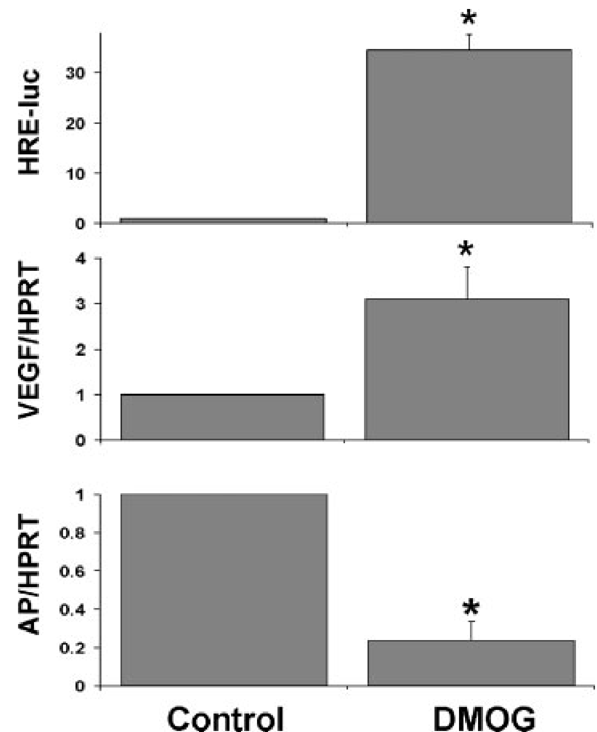

Fig. 5. Inhibition of PHD activity with DMOG treatment increases HRE reporter activity and VEGF levels while decreasing AP. MC3T3-E1 cells were grown for 14 days before receiving a 24-h treatment with 5 mM DMOG and control cells received vehicle alone. HRE transcriptional activity was measured in cells transfected with HRE-luciferase; values are expressed relative to SV40-beta-galactosidase and as a fold of control values ± SE, n = 3. RNA was isolated from other sets of osteoblasts, and VEGF and AP mRNA levels determined by RT-PCR. Levels of genes of interest were expressed relative to HPRT, a housekeeping gene that is not modulated by hypoxic conditions. Values represent averages of five separate experiments ± SE. $^*P \leq 0.05$.

levels compared to cells maintained under control normoxic conditions (Fig. 7).

DISCUSSION

It is clear that hypoxia dramatically influences cellular gene expression in most, if not all cells. While these changes are important for cellular adaptation to the environment, they can also lead to modifications in cellular function and phenotype. Hypoxia can promote cellular differentiation/lineage selection. For example, hypoxia can promote osteoclast differentiation [Arnett et al., 2003; Fukuoka et al., 2005] and mesenchymal bone marrow cell differentiation into chondrocytes [Mizuno and Glowacki, 2005; Robins et al., 2005; Schipani, 2005] and (through HIF-1 elevation) macrophage differentiation [Oda et al., 2006]. Hypoxic conditions can also suppress differentiation in cell types such as myeloid and hematopoietic cells [Sahai et al., 1994; Matsuda et al., 1998; Desplat et al., 2002; Song et al., 2002; Jiang and Mendelson, 2003] and can suppress adipogenesis and adipocyte maturation [Yun et al., 2002; Zhou et al., 2005]. Our laboratory [Ontiveros et al., 2004] and other laboratories [Tuncay et al., 1994; Park et al., 2002; Salim et al., 2004; Malladi et al., 2005] have demonstrated that osteoblast differentiation is suppressed under hypoxic conditions. Here we expanded upon our previous studies to determine the time course of

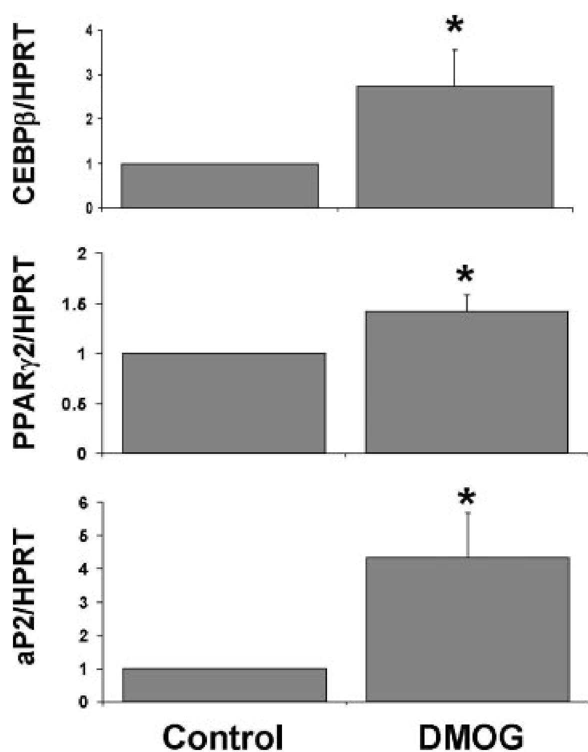

Fig. 6. DMOG increases adipocytic gene expression in MC3T3-E1 cells. MC3T3-E1 cells were grown for 14 days and then treated for 24 h with 5 mM DMOG, control cells received vehicle alone. RNA was isolated and C/EBPβ, PPARγ2, and aP2 mRNA levels were determined by RT-PCR. Levels of genes of interest were expressed relative to HPRT, a housekeeping gene that is not modulated by hypoxic conditions. Values represent averages of five separate experiments ± SE. $^*P \leq 0.05$.

gene responses and we looked at adipocyte, HIF, and PHD gene expression in response to hypoxia and PHD inhibition.

Time course analyses demonstrate a dramatic decline in AP mRNA levels that is evident within 8 h of hypoxic exposure (50% reduction) and extensive at 24 h (greater than 80% reduction). Previous studies demonstrated that AP mRNA levels were suppressed following 24 h of hypoxic exposure, but only by 10% [Park et al., 2002]. The dramatic increase in aP2 mRNA levels at 8 h suggests that osteoblasts exhibit an early response phase to hypoxia that may not require enhanced expression of C/EBPβ and PPARγ2, known upstream activators of aP2 expression. Previously, we have shown that C/EBPβ is present in osteoblasts [Iyer et al., 2004] and others have shown that PPARγ is present to some extent in osteoblasts [Maurin et al., 2005]. Alternate signaling pathways or low levels of C/EBPβ and activated PPARγ2, possibly through post-translational modification and/or ligand binding, could contribute to this response. Our results also show that as the mRNA levels of C/EBPβ and PPARγ2 increase (at 24 h and beyond), aP2 mRNA levels are further increased (Fig. 2).

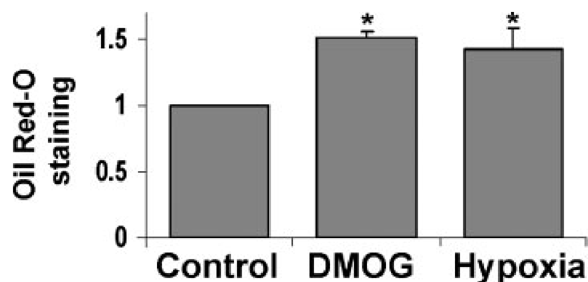

Fig. 7. Hypoxia and DMOG treatments increase lipid staining in osteoblasts. MC3T3-E1 cells were grown for 14 days and then treated with 5 mM DMOG, hypoxia, or nothing (control). After 24 h the cells were fixed and stained with oil red O. The amount of stain was quantitated and graphed. Values are averages ± SD.

While our studies indicate that adipocyte-related genes, C/EBPβ, PPARγ2, and aP2, and lipid storage function (oil-red-O staining) are induced in maturing (day 14) osteoblasts exposed to hypoxic conditions, other reports have demonstrated that hypoxia can inhibit stem cell maturation along the adipocyte lineage [Yun et al., 2002; Zhou et al., 2005; Kim et al., 2005a]. However, there are studies that indicate that exposure to hypoxia at later stages of adipocyte differentiation does not suppress adipocyte differentiation [Kim et al., 2005a]. Similarly, in preadipocytes overexpression of EPAS1, also called HIF-2α, can promote adipocyte differentiation [Shimba et al., 2004] and treatment with mitochondrial inhibitors can elevate C/EBPβ protein levels [Carriere et al., 2004]. It has been shown that mesenchymal stromal cells (hMSC-TERT) can exhibit lipogenesis under hypoxic conditions, although the full range of adipocyte markers were not expressed [Fink et al., 2004].

Our studies indicate that hypoxia suppresses the mature osteoblast phenotype and promotes development of an adipocyte-like phenotype within the exposed cell culture dish. Several mechanisms could account for these changes. One possibility is that there is a loss of mature osteoblast cells due to cell death or a reversion of cells toward an immature phenotype and an increase of immature cells developing toward an adipocytic phenotype. It is clear that increasing PPARγ activity (through PPAR agonsists or

overexpression) can promote immature bone marrow cells toward an adipocytic rather than osteoblast phenotype [Gimble et al., 2006]. Alternatively, it is possible that hypoxia is able to trigger osteoblast-adipocyte transdifferentiation. Previous studies inducing ectopic expression of the adipogenic transcription factors PPARγ and C/EBPα in MC3T3-E1 pre-osteoblasts suggest that upregulation of PPARγ in preosteoblasts is sufficient to block osteocalcin expression and increase expression of adipocyte/fat storage genes such as adipsin and aP2 [Kim et al., 2005b]. Hypoxic induction of cellular transdifferentiation has also been suggested to occur in kidney epithelial cells. After exposure to hypoxia, the kidney cells exhibit reduced expression of epithelial markers and increased expression of mesenchymal markers, suggesting an epithelial-mesenchymal transformation/transdifferentiation [Manotham et al., 2004; de Laplanche et al., 2006]. Future studies are needed to address if a similar transdifferentiation phenomenon is occurring in osteoblasts exposed to hypoxia.

Surprisingly, we found that mRNA levels of PHD2 and PHD3 were increased by hypoxic conditions. While it seems that this response would conflict with cellular responses to hypoxia, the upregulation of PHDs is thought to serve as an important negative feedback loop to regulate HIF levels [D'Angelo et al., 2003]. It may be that under conditions of excessive HIF levels osteoblasts will die. This suggests that proper titration of HIF activity may be important for the regulation of osteoblast function and survival. While HIFs levels are predominantly regulated at the post-translational level by PHDs, reports also demonstrate that mRNA levels of HIFs can be influenced by hormones [Moeller et al., 2005], prostaglandins [Critchley et al., 2006], the stage of cellular differentiation [Oda et al., 2006], tissue location [Boonyaprakob et al., 2005], and intracellular signaling pathways such as cAMP [Busca et al., 2005]. Our studies indicate that a 24-h exposure to hypoxia can induce HIF-1α but not HIF-2α mRNA levels in maturing osteoblasts. This induction might represent a response to a secreted cytokine or factor that can signal through a paracrine/autocrine mechanism to specifically influence HIF-1α but not HIF-2α transcription/mRNA levels.

Interestingly, our observed elevation of HIF-1α mRNA levels did not lead to a significant increase in HIF-1α protein levels. Previous studies have demonstrated that mRNA and protein levels of HIFs are not necessarily directly related. For example, uncoupling of oxidative phosphorylation in blastocysts leads to an increase in mRNA levels of both HIF-1α and HIF-2α mRNA levels, but only an increase in HIF-2α protein levels is observed [Harvey et al., 2004]. Consistent with our findings, others have found that HIF-2α protein levels are upregulated in MG63 osteoblast-like cells within 3 h and at 24 h of hypoxic exposure [Akeno et al., 2001], while HIF-1α protein levels were not induced under these conditions (although they could be induced by treatment with a proteosome inhibitor) [Akeno et al., 2001]. HIF-1α protein levels can be induced by hypoxia in osteocytes in vivo and in vitro [Gross et al., 2001], suggesting the possibly that in later stages of osteoblast differentiation the machinery/factors/mechanisms required for HIF-1α protein induction are present. Our cells might represent an intermediate level of maturation whereby the ability to increase HIF-2α protein levels predominates, but there is some ability to modestly increase HIF-1α protein levels.

Our studies also utilized DMOG, an inhibitor of PHDs that acts to stabilize HIFα levels at normal oxygen tensions, to examine the role of PHDs (and correspondingly HIFs) in mediating hypoxia-induced changes in osteoblast gene expression. Previous studies demonstrate the effectiveness of DMOG in elevating HIFα levels [Hanson et al., 2003] and inducing hypoxia regulated genes in other cell systems [Davidson et al., 2003; Zhao et al., 2004]. In osteoblasts, a 24-h DMOG treatment suppressed AP expression and enhanced aP2 and C/EBPβ expression (and lipid storage, Fig. 7) similar to 24 h of hypoxic conditions suggesting an important role for PHDs and HIFs in this phenotype switch (Figs. 5 and 6 versus 1 and 2). While DMOG induction of PPARγ2 was significant, it was less than what was obtained under hypoxic conditions, suggesting that additional factors such as reactive oxygen species could contribute to hypoxic induction of PPARγ2 levels.

Previous studies have demonstrated that oxygen levels can be reduced in bone through changes in arterial supply, inflammation, and diseases that decrease blood oxygen levels [Kofoed, 1986; Kiaer et al., 1988, 1992; Vogt et al., 1997; Otter et al., 1999; Harrison et al.,

2002; Szymanski et al., 2002; Ionescu and Schoon, 2003]. Conditions of reduced bone oxygenation are also associated with localized bone loss, implicating HIF activity in the regulation of bone density. Taken together, our findings suggest that exposure to hypoxic conditions can cause significant changes in osteoblast maturation and in fact can enhance lipid storage and expression of adipocyte associated genes. Modulation of PHD activity by DMOG implicates PHD and HIF activities in mediating this response.

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