HIF-1α regulates hypoxia-induced EP1 expression in osteoblastic cells

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Abstract

Changes in regional oxygen tension that occur during skeletal development and fracture stimulate local bone cell activity to regulate bone formation, maintenance and repair. The adaptive responses of bone cells to hypoxia are only beginning to be understood. The transcription factor HIF-1α is activated under hypoxia and promotes expression of genes required for adaptation and cell survival, and also regulates both bone development and fracture repair. We have previously demonstrated that hypoxic osteoblasts increase PGE_2 release and expression of the PGE_2 receptor EP1. In the present studies, we investigated the impact of altered HIF-1α activity and expression on EP1 expression in osteoblasts. HIF-1α stabilization was induced in cells cultured in 21% oxygen by treatment with DMOG or siRNA targeted against PHD2. To implicate HIF-1α in hypoxia-induced EP1 expression, osteoblastic cells were treated with siRNA targeted against HIF-1α prior to exposure to hypoxia. EP1 expression was significantly increased in cells cultured in 21% oxygen with DMOG or PHD2 siRNA treatment compared to controls. HRE activation in hypoxia was attenuated in cells treated with HIF-1α siRNA compared to controls, indicating HIF-1α as the functional HIF-α isoform in this system. Furthermore, hypoxic cells treated with HIF-1α siRNA demonstrated reduced EP1 expression in hypoxia compared to controls. Inhibition of SAPK/JNK activity significantly reduced hypoxia-induced EP1 expression but had no impact on HIF-1α expression or activity. These data strongly implicate a role for HIF-1α in hypoxia-induced EP1 expression and may provide important insight into the mechanisms by which HIF-1α regulates bone development and fracture repair.

Keywords

Bone cells respond to changes in the microenvironment by altering gene expression profile. Typically, this involves the expression of genes required for adaptation to the new conditions. A reduction in pericellular oxygen tension within bone, which occurs during

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bone development [Schipani, 2006], disuse [Dodd et al., 1999], and fracture [Brighton, 1972 #2315;Komatsu, 2004 #2051;Epari, 2008 #2314], influences bone cell phenotype. While hypoxia typically results in a switch from aerobic to anaerobic metabolism [Hochachka et al., 1996; Robin et al., 1984; Semenza et al., 1996] and a conservation of energy through global reductions in protein expression [Koumenis et al., 2002], a subset of proteins continue to be expressed, and in some cases, are upregulated. Indeed, we have recently shown that hypoxia leads to increased expression of the prostanoid receptor EP1 in osteoblasts [Lee et al., 2007].

Hypoxic regulation of gene expression generally involves activation of the hypoxia-inducible factor (HIF) transcription pathway. HIF is a heterodimer composed of an alpha and a beta subunit. Three HIF alpha subunits, identified as HIF-1α, -2α and -3α, are all capable of binding to the HIF beta subunit (HIF-1β, or aryl hydrocarbon receptor nuclear translocator [lARNT]). Both HIF-1α and HIF-2α have been implicated in osteoblastic cell responses to hypoxic conditions [Akeno et al., 2001; Dodd et al., 1999; Wan et al., 2008; Wang et al., 2007]. The stability of a HIF-α subunit is directly regulated by oxygen levels. Under normoxic conditions when molecular oxygen is present in sufficient concentrations, HIF-α is hydroxylated at proline and asparagine residues. This hydroxylation reaction prepares the HIF-α subunit for proteasomal degradation by the von Hippel-Lindau tumor suppressor protein (pVHL) [Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001] and is mediated by prolyl hydroxylase-domain proteins (PHDs 1-3). While osteoblasts express all three PHD isoforms [Irwin et al., 2007], PHD2, in particular, has the highest specific activity toward hydroxylation of HIF-1α [Hirsila et al., 2003]. Under hypoxic conditions, the constitutively expressed HIF-α subunit binds to the beta unit and translocates to the nucleus, where the HIF-α/β heterodimer binds to its DNA consensus sequence within the hypoxia responsive element (HRE) located in the promoter region of target genes [Semenza et al., 1996].

HIF stabilization and subsequent induction of target genes demonstrates potent effects on the processes of bone formation and repair. Indeed, HIF-1α expression is observed within the fetal growth plate [Schipani, 2006],, within fracture calluses up to 10 days after fracture [Komatsu and Hadjiargyrou, 2004], and in unloaded limbs [Dodd et al., 1999]. While *in vitro* data is often contradictory as to whether hypoxia is stimulatory or inhibitory for bone formation, new evidence strongly implicates hypoxia as an anabolic stimulus for bone formation [Wan et al., 2008; Wang et al., 2007]. Targeted deletion of pVHL within osteoblasts, and subsequent stabilization of HIF-α and induction of a HIF-α-responsive genetic repertoire, produced mice expressing high levels of VEGF and highly vascularized, dense long bones; in contrast, deletion of HIF-1α produced an inverse phenotype, with low levels of VEGF, poor vascularization, and thinner bones compared to wild-type mice [Wang et al., 2007]. This stimulatory effect of VHL deletion and subsequent HIF-α stabilization was not limited to skeletal development, as enhanced bone volume and vessel volume were observed during fracture repair [Wan et al., 2008]. It has even been suggested that strategies to promote HIF activity may accelerate fracture repair [Towler, 2007]. Taken together, these data suggest that an increase in EP1 expression under hypoxic conditions may be regulated by the HIF pathway and could play an important role in bone repair.

Members of the mitogen-activated protein kinase (MAPK) signal transduction pathway are also activated in response to hypoxia [Matsuda et al., 1998], including stress-activated protein kinases (SAPKs) [Seko et al., 1997], which have been shown to regulate hypoxia-induced gene expression. For example, SAPKs have been shown to stabilize *VEGF* mRNA to enhance its expression during hypoxia [Pages et al., 2000]. The present study was designed to investigate the impact of HIF-1α and MAPKs on the regulation of the PGE_2 receptor EP1 during hypoxia. MC3T3-E1 osteoblastic cells were cultured under hypoxic conditions (2% oxygen) for 24 hours and the role of HIF-1α, PHD2, and MAPKs in hypoxia-induced EP1 expression was investigated. We demonstrate herein that hypoxia and hypoxia mimetics increase EP1 transcript and protein product, and that HIF-1α siRNA attenuates hypoxia-induced EP1 expression. We further demonstrate that siRNA reductions of PHD2 increase both HIF-1α and EP1 expression under normoxic conditions, and that increased EP1 expression under hypoxia requires SAPK/JNK activity. These data highlight a possible mechanism to explain the reported effects of hypoxia on bone formation and repair.

Materials & Methods

Cell Culture

MC3T3-E1 clone 14, which are well-characterized murine osteoblastic cells, (ATCC), were cultured at a density of 10,000 cells/cm^2 in 10 cm petri dishes in Minimum Essential Medium, alpha modification (α-MEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P&S). For ambient (21%) oxygen tension experiments, cells were cultured in a standard humidified incubator at 37°C with a 95% air and 5% CO_2 atmosphere. For hypoxic (2%) oxygen tension experiments, cells were cultured in humidified incubators at 37°C with 5% CO_2 with oxygen tension reduced using supplemental N_2 (HERAcell® 150, Kendro). For experiments, reduced serum media was used containing α-MEM, 0.1% FBS and 1% P&S that had been pre-conditioned in 2% or 21% oxygen for 24 hours. To determine the effects of HIF activation independent of hypoxia on EP1 expression, cells cultured in 21% oxygen were treated with 1mM dimethyloxaloglycine (DMOG; BIOMOL International L.P.), a known activator of the HIF pathway [Hanson et al., 2003].

RNA extraction and quantitative PCR

Total RNA was collected using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions, and included on-column digestion of genomic DNA. 0.2-1 μg of total RNA was reverse-transcribed with Superscript II Reverse Transcriptase (Invitrogen). qtPCR was performed using TaqMan® Universal PCR Master Mix (Applied Biosystems) on a Mastercycler® realplex2 (Eppendorf); all primers and probes were purchased from Applied Biosystems. Amplification conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Quantitative PCR results were first normalized to *ActB* transcript level to yield C_t, then normalized to control conditions (*e.g.*, normoxia at the same time point) to generate ΔC_t. Fold change in expression was subsequently calculated using the formula $2^{-\Delta C_t}$ [Livak and Schmittgen, 2001].

Western immunoblotting

Whole cell protein lysates were collected in RIPA homogenization buffer (1% Igepal CA-630, 0.5% sodium deoxycholate and 0.1% SDS in PBS). Protein concentration was determined using the DC Protein Assay (Bio-Rad). Equal amounts of protein (20 μg) were loaded into SDS-polyacrylamide gels, resolved by electrophoresis and transferred to nitrocellulose membranes. For detection of HIF-1α, membranes were blocked in 0.5% non-fat milk in TBST (Tris-buffered saline with 0.05% Tween-20) for 2 hours, followed by overnight incubation at 4°C with a 1:200 dilution of an anti- HIF-1α antibody (Cayman Chemicals). For detection of EP1 and PHD2, membranes were blocked in 5% non-fat milk in TBST for 2 hours, followed by an overnight incubation at 4°C with a 1:400 EP1 (Cayman Chemicals) antibody dilution or a 2 hour incubation with a 1:1000 dilution of an anti-PHD2 antibody (Novus Biologicals). Following three washes in TBST, membranes were incubated with appropriate secondary antibodies linked to horseradish peroxidase (HRP; Jackson ImmunoResearch Laboratories) at 1:5,000 dilution for 1 hour. After further washing in TBST and then PBS, the membranes were soaked in ECL detection reagents for 1 minute (Amersham) and then exposed to X-ray film. To confirm equal loading of protein, the membranes were stripped and probed for expression of β-actin at a dilution of 1:2,500 (Abcam) or α-tubulin at a dilution of 1:2,500 (Cell Signaling). Densitometry was perfomed using QuantityOne (Bio-Rad).

RNA silencing of HIF-1α and PHD2

Small interfering RNAs (siRNA) were transfected into osteoblastic cells to silence expression of HIF-1α and PHD2. For HIF-1α (GenBank™ accession No. NM_010431), cells were transfected with 50 nM of siRNA targeted for the sequence CCG CTC AAT TTA TGA ATA TTA (Qiagen). After 48 hours, cells transfected with HIF-1α siRNA were transferred to hypoxic conditions for an additional 24 hour incubation, followed by collection of whole cell lysates or total RNA. For PHD2 (GenBank™ accession No. NM_053207), cells were transfected with 80 nM of siRNA targeted for the sequence ATG CGT GAC ATG TAT ATA TTA (Qiagen). Cells transfected with PHD2 siRNA were cultured for 96 hours in 21% oxygen, followed by collection of whole cell lysates. For all siRNA experiments, cells were transfected using Hiperfect transfectant reagent (Qiagen) at conditions optimized for the cell line and in reduced serum experimental media. To assess transfection efficiency and target sequence specificity, cells were transfected with the fluorescein-conjugated control scrambled siRNA sequence for the target sequence AAT TCT CCG AAC GTG TCA CGT (Qiagen). As an additional control, some cells were treated only with Hiperfect.

Transient HRE expression assay

To assess the impact of HIF-1α silencing on HRE activation, cells that were transfected with HIF-1α or control siRNA were again transfected 24 hours later with a pGL3-HRE DNA reporter plasmid vector containing the HRE (AGCGTG) binding site, and a Renilla luciferase vector, pRL-TK (Promega) using Lipofectamine™ 2000 (Invitrogen) as previously described [Lee et al., 2007] and exposed to 2% oxygen for 24 hours. Cell lysates

were collected and luciferase activity levels were measured using the Dual-Luciferase® Reporter Assay System (Promega) and a Turner Designs Model 20/20 luminometer.

Inhibition of MAP kinase activity

To investigate the role of MAPKs on hypoxia-induced EP1 expression, MEK, p38, and JNK were inhibited using well-characterized pharmacologic antagonists. Twenty hours prior to the start of the experiment, cells were seeded at a density of 10,000 cells/cm^2 in normal growth media. The cells were treated with either 10 μM U0126 (MEK inhibitor; Cell Signaling), 20 μM SB203580 (p38 inhibitor; Calbiochem), or 10 μM SP600125, (JNK inhibitor; Calbiochem) for one hour prior to and during a 24 hour exposure to hypoxia. Control cells were treated with 0.1% DMSO as appropriate (vehicle control) one hour prior to and during the 24 hours. Whole cell lysates were collected, and EP1 protein levels were analyzed by western blot as described above.

Statistical analysis

To compare differences in HIF-1α, EP1, and PHD2 protein expression between groups, one-way analysis of variance (ANOVA) was used with a Dunn's multiple comparison post-hoc test when appropriate. An unpaired *t*-test was used to assess differences in EP1 mRNA levels. Protein densitometry and EP1 transcript were normalized to expression at 21% oxygen to reduce sample-to-sample variability. α-tubulin and β-actin were used interchangeably as loading controls; we have confirmed no influence of hypoxia on transcript or protein expression for either (data not shown). *P* < 0.05 was considered statistically significant.

Results

Effect of hypoxia and hypoxia mimetics on EP1 expression

Western immunoblotting and qtPCR were used to quantify hypoxic regulation of EP1 at the level of protein and transcript. MC3T3-E1 osteoblasts cultured for 24 hours at 21% oxygen expressed little EP1 protein (Figure 1A). In contrast, osteoblasts that were cultured at 2% oxygen, or at 21% oxygen in the presence of DMOG, an activator of the HIF signaling pathway, expressed greater levels of EP1 protein compared to normoxic controls (Figure 1A). Densitometric analysis of EP1 protein levels compared to β-actin levels revealed a 3.4- and 4.8-fold increase in EP1 expression relative to normoxic controls in cells cultured at 2% oxygen or 21% oxygen in the presence of DMOG, respectively (Figure 1B). There was no significant difference in EP1 protein expression in cells cultured at 21% oxygen in the presence of DMOG compared to those cultured at 2% oxygen.

Because reductions in pericellular oxygen are associated with stabilization of HIF-α and subsequent induction of HIF-α target genes, we next sought whether EP1 transcript levels were increased under hypoxia compared to normoxic controls. MC3T3-E1 osteoblastic cells cultured at 2% oxygen for 24 hours demonstrated a 2.5-fold increase in EP1 transcript compared to normoxic controls (Figure 2). That DMOG, an activator of the HIF pathway, successfully mimicked the influence of 2% oxygen on EP1 expression, suggests a role for

HIF in hypoxia-induced EP1 expression; this is strengthened by the observation that hypoxia increased EP1 transcript.

Effects of HIF-1α siRNA on hypoxia-induced EP1 expression

To further investigate the role of HIF-1α activation in hypoxia-induced EP1 expression, cells were transfected with siRNA targeted against HIF-1α. Cells that were transfected with siRNA directed against HIF-1α demonstrated attenuated HIF-1α levels under hypoxia compared to cells transfected with a scrambled, non-silencing siRNA (Figure 3A). These same HIF-1α transfected cells expressed lower EP1 protein compared to control cells (Figure 3A), and this reduction in EP1 expression was determined to be statistically significant by densitometry (Figure 3B). This data confirms a role for HIF-1α activation in hypoxia-induced EP1 expression.

Effects of silencing HIF-1α on HRE activation

We have previously shown that hypoxia activates the HRE using a HRE-luciferase expression vector [Lee et al., 2007]. To assess the contribution of HIF-1α to HRE activation, cells transfected with HIF-1α siRNA were co-transfected with the HRE-luciferase expression vector. HRE luciferase activity in cells cultured at 2% oxygen was significantly increased compared to cells transfected with HIF-1α siRNA and cells grown at 21% oxygen. There was no statistical difference in HRE activity between cells cultured in 21% oxygen and cells transfected with HIF-1α siRNA and grown in 2% oxygen (Figure 4). These data confirm that HIF-1α is the functional alpha subunit responsible for HRE activation in these cells.

The influence of PHD2 on HIF-1α and EP1 expression under hypoxia

Ubiquitination and degradation of HIF-α isoforms involves their hydroxylation at proline residues 406 and 564, which generates binding sites for the Von Hippel Landau ubiquitin ligase; hydroxylation at Pro^{406} and Pro^{564} is mediated by the prolyl hydroxylase domain (PHD) proteins, of which there are four isoforms (PHD1-4). To assess the involvement of PHD2 in HIF-1α activation and EP1 expression, cells were transfected with PHD2 siRNA, control siRNA or Hiperfect alone. Cells transfected with siRNA directed against PHD2 and cultured at 21% oxygen demonstrated a modest reduction in PHD2 protein levels, yet a large increase in both HIF-1α and EP1 protein levels compared to controls (Figure 5). These data indicate that PHD2 is involved in the suppression of HIF-1α and control of EP1 expression in osteoblastic cells cultured in 21% oxygen.

Influence of MAP kinases on hypoxia-induced EP1 expression

Induction of gene transcription in response to hypoxic conditions can involve the activation of MAP kinases, including stress-activated protein kinase (SAPK/JNK), which is involved in stabilization of VEGF transcript [Pages et al., 2000]. To investigate the role of MAPKs in hypoxic regulation of EP1, cells were pre-treated with MAPK inhibitors or vehicle control for 1 hour prior, and maintained during, hypoxic culture. Inhibition of MEK or p38 had no effect on hypoxia-induced increased expression of EP1 in MC3T3-E1 cells as determined by Western blot analysis. In contrast, SAPK/JNK inhibition abrogated hypoxia-induced EP1

protein expression compared to cells treated with vehicle (Figures 6A and 6B). Inhibition of SAPK/JNK demonstrated no effect upon hypoxia-induced HIF-1α expression (data not shown), nor did it influence HRE-luciferase activity compared to hypoxic vehicle controls (Figure 7), suggesting that SAPK/JNK functions downstream of HIF induction of EP1 transcript.

Discussion

Activation of the HIF pathway is a mechanism commonly used by cells to regulate gene expression in response to hypoxia. HIF signaling involves the formation of a HIF-α/β complex, which translocates into the nucleus where it binds to its consensus sequence within the promoter region of target genes [Caro, 2001]. Previously, we demonstrated this pathway to be activated in MC3T3-E1 osteoblastic cells exposed to 2% oxygen, and that these same cells had increased EP1 protein levels compared to cells cultured in 21% oxygen [Lee et al., 2007]. In this paper, we demonstrate that HIF-1α is required for hypoxia-induced EP1 expression in osteoblastic cells. To our knowledge this is the first report of HIF-1α regulation of any prostanoid receptor.

DMOG was used as a tool to activate the HIF pathway under non-hypoxic conditions (21% oxygen). Cells cultured in 21% oxygen treated with DMOG had increased EP1 protein levels compared to cells cultured in 21% oxygen without DMOG confirming a role for the HIF pathway in regulation of EP1 expression. Using siRNA targeted for HIF-1α RNA, we effectively reduced HIF-1α protein levels by approximately 86% in hypoxic cells, which nearly completely abrogated hypoxia-induced EP1 expression (Figure 3). In addition, silencing HIF-1α protein expression attenuated hypoxia-induced HRE activation to levels similar to that of cells cultured in 21% oxygen (Figure 4), thus indicating HIF-1α is the alpha subunit responsible for HRE activation in this system. Furthermore, even a modest reduction in PHD2 expression using siRNA, stabilized HIF-1α levels and enhanced EP1 protein expression in 21% oxygen (Figure 5). Taken together, these data strongly support a role for HIF-1α in hypoxia-induced EP1 expression.

Mitogen-activated protein kinases (MAPK) regulate many events in cell biology including protein translation and activation of transcription factors such as HIF-1α [Minet et al., 2001]. Data exist to support a relationship between MAPK activity and HIF-1α activation. For example, increased SAPK/JNK activity has been observed in hypoxia and shown to promote HIF-1α activation in certain cell types [Choi et al., 2006; Comerford et al., 2004]. In addition, hypoxia-induced ERK1/2 and p38 activity has been shown to phosphorylate and activate HIF-1α [Conrad et al., 1999; Minet et al., 2001; Raymond and Millhorn, 1997; Sodhi et al., 2000]. In our study, inhibition of the MAPKs ERK1/2, and p38 had no impact on hypoxia-induced EP1 expression. However, SAPK/JNK inhibition reduced hypoxia-induced EP1 expression by approximately 67%. While our data reveal a role for SAPK/JNK in hypoxia-induced EP1 expression, inhibition of SAPK/JNK activity had no impact on HRE activation in our system. This data suggests that involvement of SAPK/JNK in hypoxia-induced EP1 expression may occur independently or downstream of HIF-1α.

In this paper, we demonstrated for the first time that HIF-1α regulates hypoxia-induced EP1 protein expression. Cells respond to hypoxia by reducing protein synthesis, beginning at the level of translation followed by regulation at the level of transcription [Pettersen et al., 1986]. Due to the high metabolic cost of protein synthesis, a reduction may be critical for adaptation to the new environment with reduced oxygen. This begs the question of why EP1 expression is markedly induced, and suggests EP1 may have an important role in osteoblast survival or adaptive responses to hypoxic conditions.

Bone formation occurs under conditions of low oxygen tension. During embryogenesis, endochondral and intramembranous ossification occur near capillary ingrowth and angiogenesis. Reductions in pericellular oxygen are also observed as a result of disuse and fracture. It is possible that hypoxia-induced EP1 expression may be a response of osteoblast cells to promote bone formation, remodeling and repair under these conditions. EP1 has been detected in the growth plate, a region previously identified as hypoxic, and regulated by changes in oxygen tension [Schipani, 2006]. For example, EP1 has been shown to be strongly expressed in the proliferative and hypertrophic zones of the growth plate [Brochhausen et al., 2006]. Activation of EP1 in growth plate chondrocytes promotes proliferation *in vitro* [Epstein et al., 2001], and therefore may regulate cell physiology within the growth plate *in vivo*. Moreover, evidence suggests that PGE_2 induces osteoblast fibronectin expression, a protein involved in the early stages of bone formation, via activation of the EP1 receptor [Tang et al., 2005]. Activation of EP1 is also involved in PGE_2-induced osteoblast cell proliferation [Suda et al., 1996] and in PGE_2-induced osteoclastogenesis [Tsujisawa et al., 2005]. PGE_2 levels are elevated at a fracture site [Dekel et al., 1981], and evidence suggests that PGE_2 and cyclooxygenase-2 are crucial for fracture repair [Simon et al., 2002; Zhang et al., 2002]. These data support a potentially critical role for PGE_2 signaling through EP1 under hypoxic conditions. Interestingly, mice with constitutively active HIF-1α expression in osteoblasts have been shown to produce more bone in a distraction osteogenesis model [Wan et al., 2008]. Our data showing HIF-1α regulation of EP1 receptor levels in osteoblasts may provide insight into the mechanisms by which HIF regulates both bone development and fracture repair.

Acknowledgments

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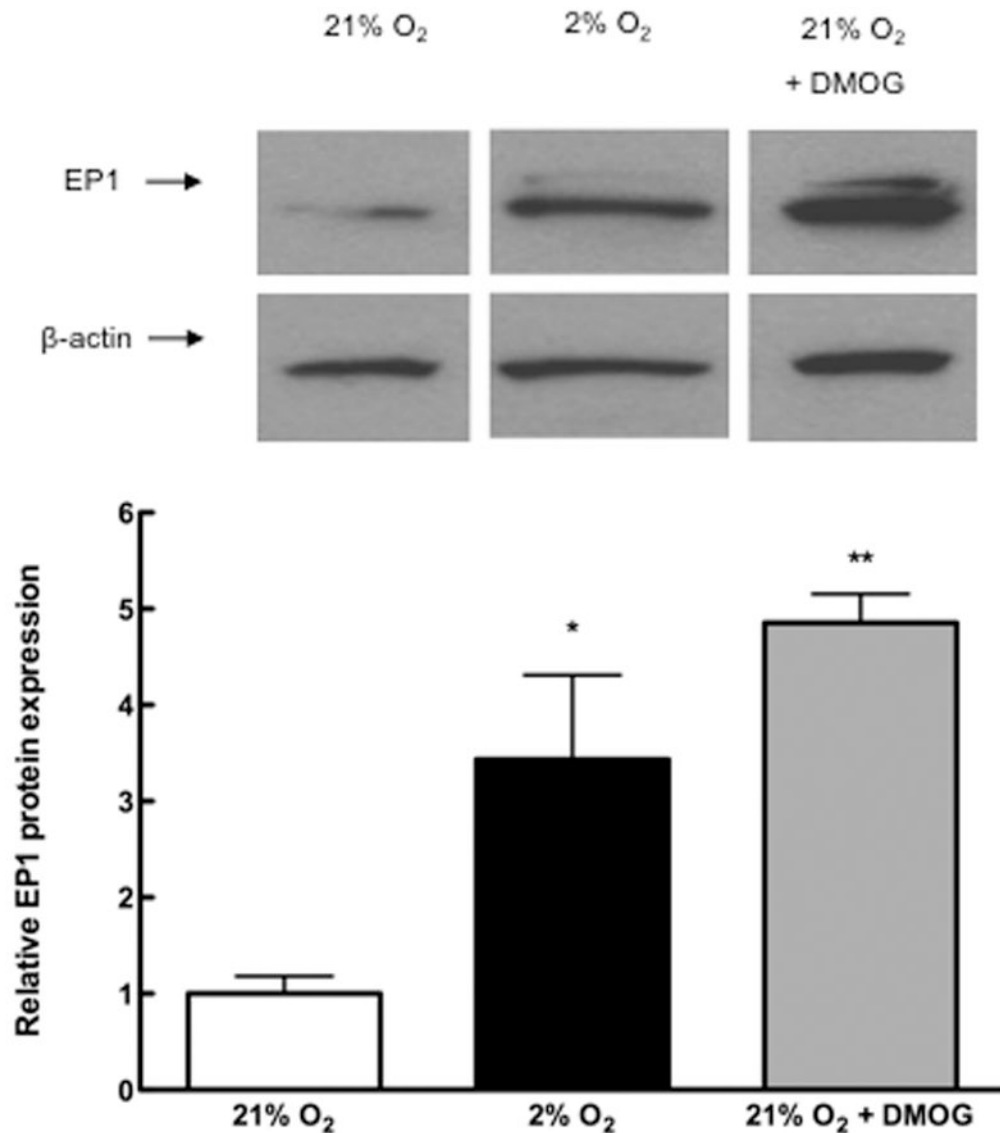
Figure 1. Effects of hypoxia and HIF activation on EP1 protein levels in MC3T3-E1 cells
(A) Representative Western blots for EP1 protein levels in cells cultured for 24 hours under normoxia (21% O_2), hypoxia (2% O_2), or normoxia plus PHD inhibitors (21% O_2 + DMOG). Membranes were probed for β-actin as an internal control for equal protein loading, n=3. (B) Densitometry data of EP1 protein levels normalized to β-actin and 21% O_2 control. Bars represent mean + SEM; * and ** denote $p < 0.05$ or $p < 0.01$ compared to 21% O_2, respectively. n=3.
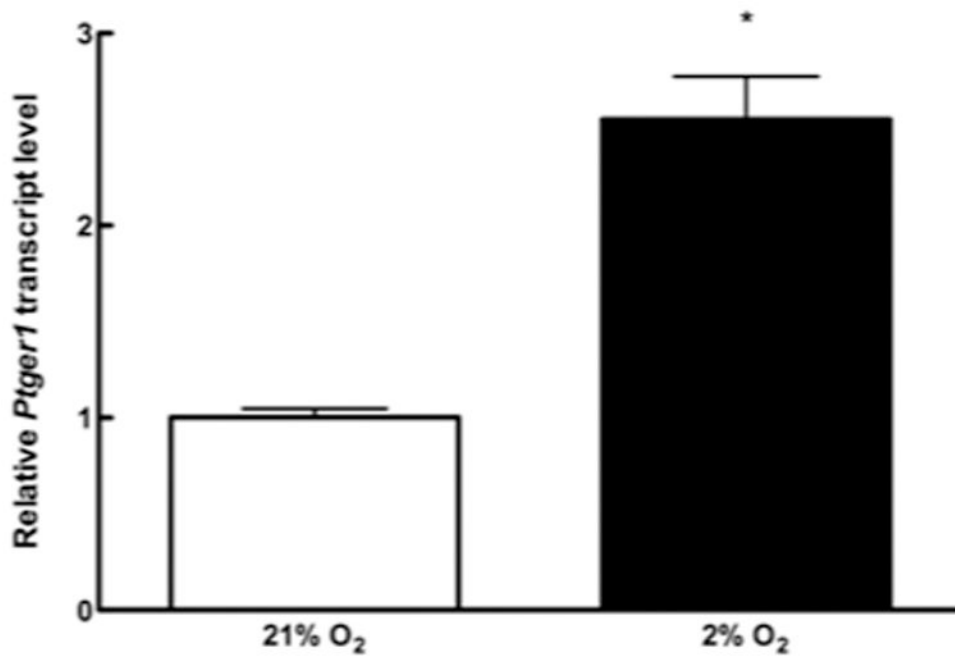
Figure 2. Effect of hypoxia on EP1 transcript in MC3T3-E1 cells
Total RNA was collected from MC3T3-E1 osteoblasts cultured at 21% or 2% O_2 for 24 hours, reverse-transcribed into cDNA, and quantitatively examined for EP1 (*Ptger1*) and β-actin (*ActB*) expression. Bars represent mean + SEM; ** denotes $p < 0.01$ compared to 21% O_2, respectively. n=3.
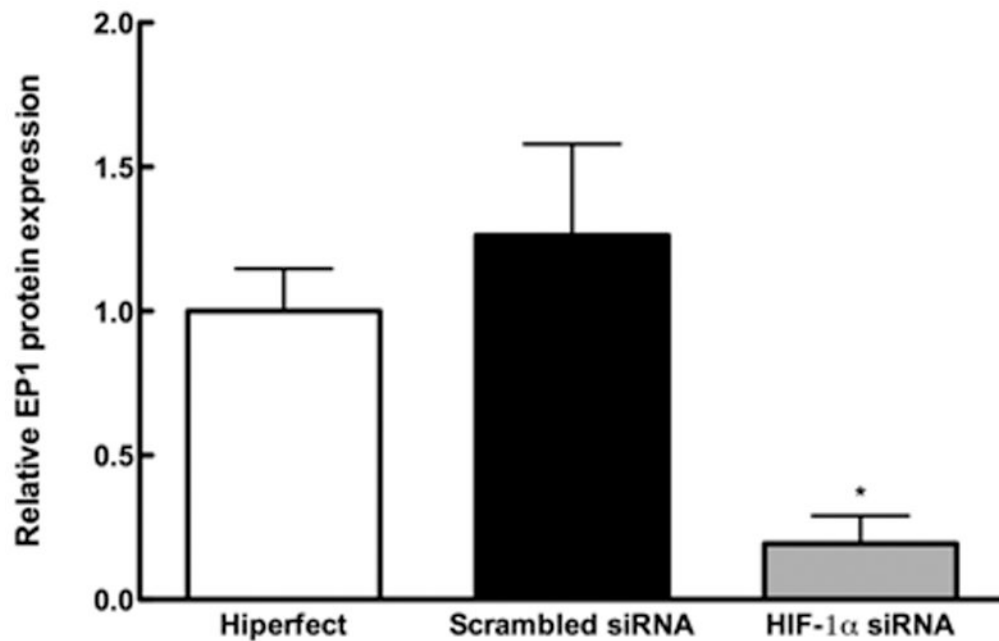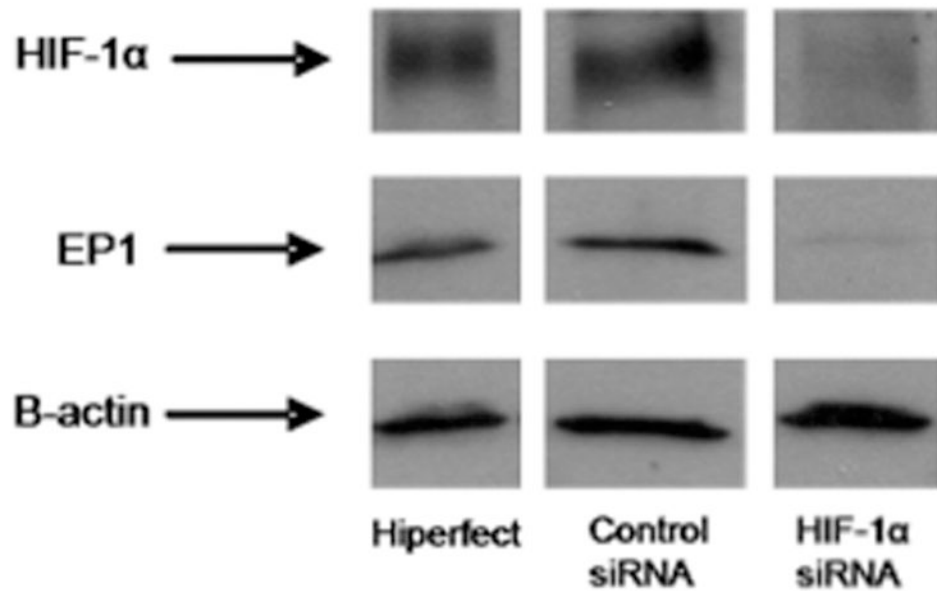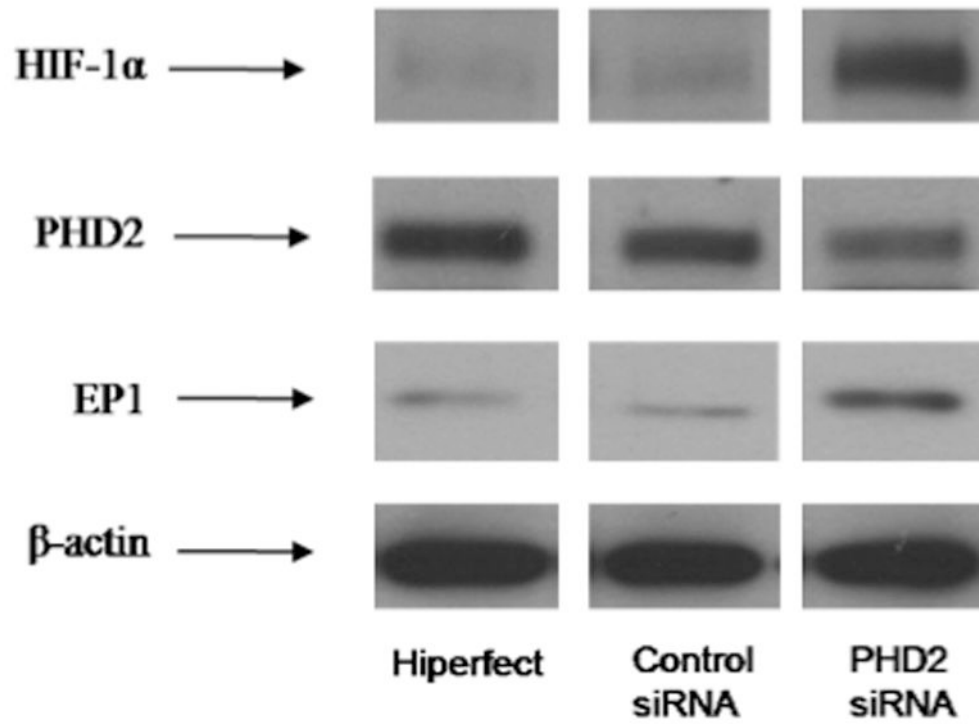
Figure 3. Effects of HIF-1α siRNA on EP1 protein levels in MC3T3-E1 cells exposed to hypoxia
(A) Representative Western blots for HIF-1α and EP1 protein levels in cells treated with
Hiperfect alone or transfected with either HIF-1α or control siRNA followed by 24 hours
exposure to 2% oxygen. β-actin was used as a loading control, n=6. (B) Quantitation of EP1
protein levels normalized to β-actin and Hiperfect control at 2% oxygen. Bars represent
mean + SEM, * denotes statistically significant difference compared to Hiperfect or
scrambled siRNA, p<0.05, n=6.
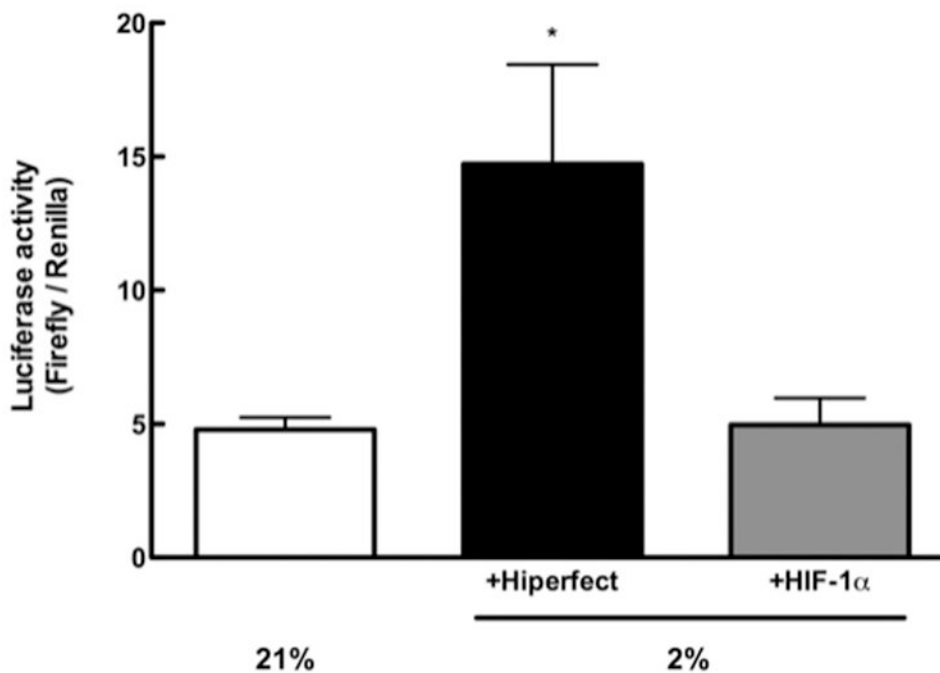
Figure 4. Effects of HIF-1α siRNA on expression of pGL3-HRE vector in MC3T3-E1 cells
Control cells or cells transfected with HIF-1α siRNA were exposed to 2% or 21% oxygen
for 24 hours after which luciferase activity levels were measured. Bars respresent mean
luciferase activity $\pm$ SEM, expressed as relative luciferase units (RLU), and normalized to a
Renilla luciferase internal control. * denotes a statistically significant difference compared
to 21% oxygen, $p<0.05$, n=3.

**Figure 5. Effects of PHD2 siRNA on HIF-1α and EP1 protein levels**
Representative Western blots from cells transfected with Hiperfect alone (transfectant
control), PHD2 siRNA, or control siRNA, and cultured in 21% oxygen for 96 hours. β-actin
was used as a loading control. n=3
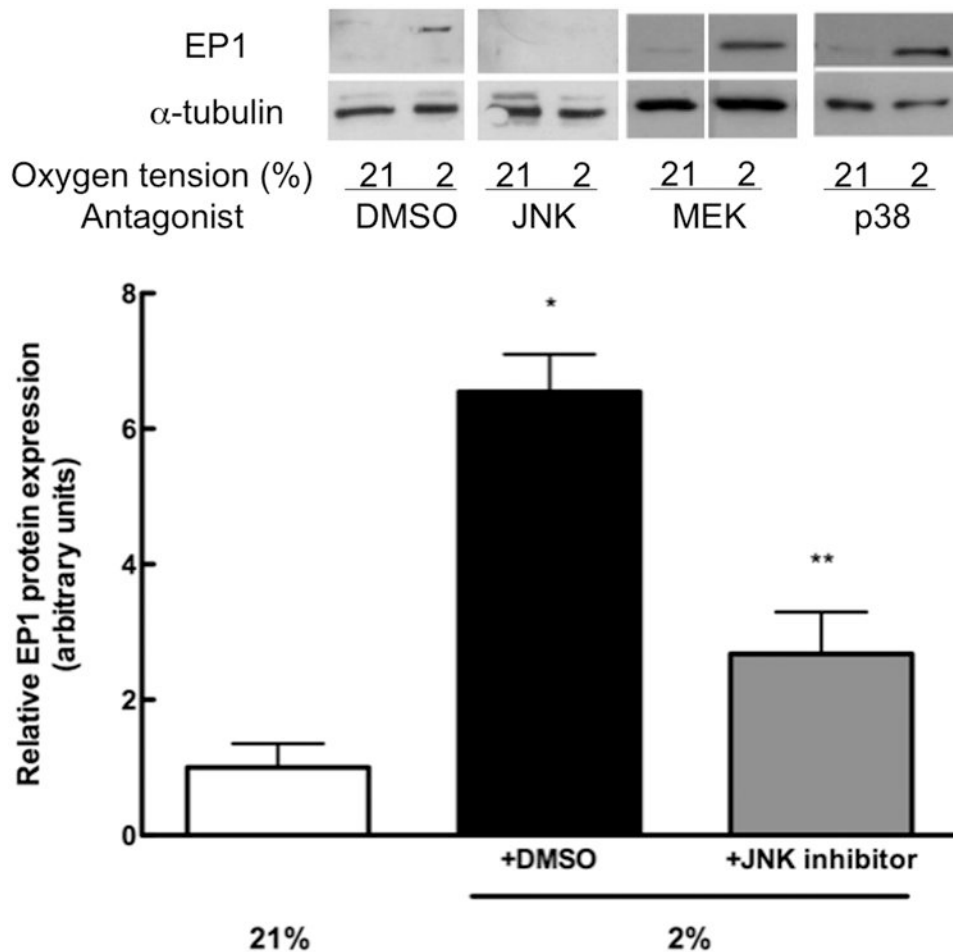
Figure 6. Inhibition of SAPK/JNK activity prevents EP1 expression under hypoxia
(A) Representative Western blots for EP1 protein levels in cells treated with either 10 µM SP600125 (JNK inhibitor) or 0.1% DMSO and cultured for 24 hours in 2% oxygen, or in 21% oxygen with 0.1% DMSO. α-tubulin was used as a loading control, n=4. (B) Densitometric analysis of EP1 expression normalized to α-tubulin. Bars represent mean + SEM, * and ** denotes statistically significant difference compared to cells cultured in 21% oxygen with DMSO or 2% oxygen with DMSO, respectively, p<0.05, n=3.
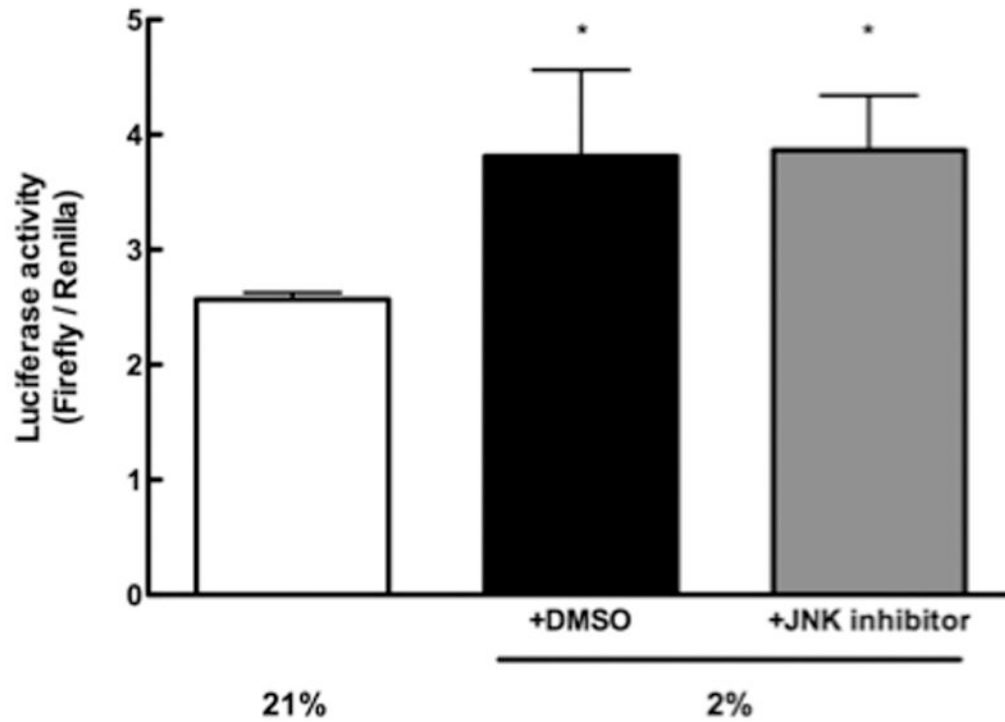
Figure 7. Effect of SAPK/JNK inhibition on pGL3-HRE luciferase

MC3T3-E1 cells transfected with an HRE luciferase expression vector, were treated with 10 μMSP600125, JNK inhibitor, or 0.1% DMSO (vehicle control). Bars represent mean luciferase activity $\pm$ SEM, * denotes statistically significant difference compared to 21% oxygen, p<0.05, n=4.