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Stratified control of IGF-I expression by hypoxia and stress hormones in osteoblasts

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Abstract

Bone cells respond to the integrated effects of local and systemic regulation. Here we show that hypoxia and the stress hormones PGE₂ and glucocorticoid interact in complex ways in osteoblasts, converging on insulin like growth factor I (IGF-I) expression. Whereas hypoxia alone rapidly increased transcription factor HIF activity, it suppressed DNA synthesis, had no significant effects on protein synthesis or alkaline phosphatase activity, and drove discrete changes in a panel of osteoblast mRNAs. Notably, hypoxia increased expression of the acute phase response transcription factors C/EBP δ which can induce IGF-I in response to PGE₂, but conversely prevented the stimulatory effect of PGE₂ on IGF-I mRNA. However, unlike its effect on C/EBP δ , hypoxia suppressed expression of the obligate osteoblast transcription factor Runx2, which can activate an upstream response element in the IGF-I gene promoter. Hypoxic inhibition of IGF-I and Runx2 were enforced by glucocorticoid, and continued with prolonged exposure. Our studies thus reveal that IGF-I expression is stratified by two critical transcriptional elements in osteoblasts, which are resolved by the individual and combined effects of hypoxic stress and stress hormones. In so doing, hypoxia suppresses Runx2, limits the enhancing influence of PGE₂, and interacts with glucocorticoid to reduce IGF-I expression by osteoblasts.

Keywords

PGE₂; glucocorticoid; HIF; C/EBP; Runx2

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1. INTRODUCTION

The growth factor insulin-like growth factor I (IGF-I) critically controls bone development and remodeling, and its own expression is regulated within the skeleton by other bone growth factors and hormones (McCarthy and Centrella, 2001; Centrella et al., 2004). Early studies in osteoblasts showed that any agent, including the locally synthesized stress hormone prostaglandin E₂ (PGE₂), that can increase intracellular cyclic AMP (cAMP) and activate protein kinase A (PKA), rapidly induces IGF-I mRNA levels. This occurs in large part through the synthesis, activation and nuclear translocation of the abundant transcription factor CCAAT enhancer binding protein δ (C/EBP δ), and to a lesser extent C/EBP β , and their association with a *cis*-acting response element (RE) within exon 1 of the IGF-I gene near the start site of transcription (McCarthy et al., 1995; Thomas et al., 1996; Umayahara et al., 1997; Umayahara et al., 1999; McCarthy et al., 2000b; Billiard et al., 2001). This differs from a direct inhibitory effect on IGF-I mRNA levels by persistent exposure to the circulating stress hormone glucocorticoid (McCarthy et al., 2000b). Curiously, glucocorticoid rapidly increases the expression of C/EBP δ and C/EBP β , but in this instance the C/EBPs remain in the cytoplasm until subsequent activation by, for example, the cAMP inducer PGE₂ (McCarthy et al., 2000b). Indeed, rapid changes in C/EBPs are clearly linked to the acute phase response and are thought to control the expression of genes associated with downstream reaction or repair mechanisms (Akira et al., 1990; Brasier et al., 1990a; Brasier et al., 1990b). By contrast to its stimulatory effect on expression of the C/EBPs, however, glucocorticoid potently decreases the level of the essential osteogenic *runt* homology domain transcription factor Runx2 (Chang et al., 1998). Therefore, normal diurnal variations in serum glucocorticoid may permissively maintain local IGF-I expression in combination with other hormone signals that activate the C/EBPs, but persistent pathologic or pharmacologic exposure to glucocorticoid may cause bone wasting effects at least in part through loss of Runx2.

Whereas a complex balance between the local or systemic stress hormones PGE₂ and glucocorticoid may influence net IGF-I expression and osteogenesis, we hypothesized that some stress systems could stratify the response to these hormones in specific ways. Among the many stressors that influence skeletal integrity, variations in local oxygenation could significantly alter the metabolic activity of resident bone cells. In this context, bone forming osteoblasts that transition through various zones of the bone microenvironment during the process of differentiation experience changes in ambient oxygen. Some studies indicate oxygen levels as low as 1–7% within the bone marrow and as such have an important regulatory influence on the survival and activity of resident mesenchymal stem cell populations. By comparison, cells found closer to the bone surface or adjacent to vascular blood supply are thought to experience significantly higher oxygen levels (Utting et al., 2006; Wang et al., 2007a; Wang et al., 2007b; Wan et al., 2008a; Wan et al., 2008b; Zahm et al., 2008; Arnett, 2010; Eliasson and Jonsson, 2010; Zahm et al., 2013). Differences in ambient oxygen may therefore initially be perceived as a stressor until the cells adapt to a new set point. In this study we asked if variations in ambient oxygen alone controlled IGF-I expression by osteoblasts, and whether the response of these cells to PGE₂ and glucocorticoid differed between normoxic and hypoxic conditions.

2. MATERIALS AND METHODS

2.1 Cells and treatments

Primary osteoblast-enriched cultures were isolated from parietal bones of 22 day old Sprague-Dawley rat fetuses (Charles River Laboratories), as approved by Yale Institutional Animal Care and Use Committee. Sutures were dissected and cells were released by 5 sequential collagenase digestions. Cells from the last 3 digestions express features of differentiating osteoblasts, including high levels of transcription factor Runx2, parathyroid hormone receptor, type I collagen synthesis, and alkaline phosphatase. They also increase osteocalcin expression in response to dihydroxyvitamin D3, display differential sensitivity to transforming growth factor β (TGF- β), bone morphogenetic protein 2, and various PGs, and form mineralized nodules under conditions that promote long term differentiation *in vitro* (Centrella et al., 1987; McCarthy et al., 1988; Centrella et al., 1994; Centrella et al., 1995; Centrella et al., 1996; Ji et al., 1997; Carpenter et al., 1998; Ji et al., 1998). Cells were plated at 4,000/cm² in Dulbecco's modified Eagle's medium supplemented with 100 μ g/ml ascorbic acid, nonessential amino acids, antibiotics, and 10% fetal bovine serum, and grown for at least 6 days before transfection or treatments. To produce a hypoxic environment, post-confluent cell cultures were incubated in a humidified sealed chamber infused with 1% oxygen. Hypoxic cultures were incubated in parallel with control cultures under a normoxic state of 21% oxygen. To compare the effect of hypoxia and normoxia on *de novo* IGF-I expression, post-confluent cell cultures were treated with vehicle or 1 μ M PGE₂ for the last 6 hours of incubation. To determine the influence of glucocorticoid, post confluent hypoxic and normoxic cell cultures were treated with vehicle or 100 nM cortisol before vehicle or PGE₂ dependent induction of IGF-I.

2.2 Plasmids

Hypoxia induced transcription factor (HIF) dependent gene expression was assessed with a firefly luciferase reporter plasmid containing five consensus HIF RE (Yun et al., 2005). IGF-I gene promoter activity was assessed with a luciferase reporter plasmid driven by a 1.7 kb fragment of the promoter and 0.3 kb fragment of exon 1 cloned from the rat IGF-I gene (McCarthy et al., 1997; McCarthy et al., 2000b; McCarthy et al., 2000c). C/EBP dependent gene expression was assessed with a luciferase reporter plasmid containing four C/EBP RE derived from the native IGF-I promoter (McCarthy et al., 2000b; McCarthy et al., 2000c). Runx dependent gene expression was assessed with luciferase reporter plasmid containing two copies of a Runx RE from the rat TGF- β receptor I gene promoter (Ji et al., 1998; Zawel et al., 1998; Ji et al., 2001; McCarthy, 2003). HIF1 α and HIF2 α gene promoter fragments fused to firefly luciferase were cloned from human DNA by PCR amplification as described (Lin et al., 2011). Glucocorticoid dependent gene expression was assessed with a luciferase reporter plasmid containing a well characterized glucocorticoid RE from the mouse mammary tumor virus (Chang et al., 1998; McCarthy, 2003). C/EBP δ and Runx2 transcriptional activation were assessed with expression plasmids encoding full length C/EBP δ or Runx2 fused to the DNA binding domain (DBD) of yeast transcription factor GAL4 (GAL4 DBD-C/EBP δ and GAL4 DBD-Runx2), and luciferase reporter plasmid containing five GAL4 REs (5XGAL4) (McCarthy et al., 2000c; McCarthy, 2003; Centrella, 2004; McCarthy et al., 2007). Endogenous Runx2 levels were enhanced with an expression

plasmid encoding mouse Runx2 and endogenous C/EBP δ levels were enhanced with an expression plasmid encoding rat C/EBP δ (McCarthy et al., 2000c; Ji et al., 2001; McCarthy, 2003; McCarthy et al., 2007).

2.3 Transfections

Reporter plasmids, expression plasmids, and empty vector plasmids were pre-titrated for optimal expression efficiency and transfected with reagent TransIT LT1 (Mirus). Cell cultures at least 80% confluent (~40,000 cells per cm²) were exposed to 25 ng per cm² of expression plasmid DNA and/or 37.5 to 75 ng per cm² of reporter plasmid DNA for 16 h or longer in medium containing 4% fetal bovine serum. Cells were then treated as indicated in each figure in serum free medium. Cells were lysed and extracts were analyzed by comparison to positive and negative control plasmids, and where appropriate, corrected for differences in total protein content (McCarthy et al., 2000c; Ji et al., 2001; McCarthy, 2003; McCarthy et al., 2007).

2.4 DNA synthesis

Cells were incubated with 5 μ Ci/ml [*methyl*-³H]thymidine (80 Ci/mmol) for the last two hours of culture. Cells were lysed in 0.1 M sodium dodecyl sulfate and 0.1 M sodium hydroxide, collecting the precipitate formed with 10% trichloroacetic acid, and scintillation counting (Centrella et al., 1987; Centrella et al., 1995).

2.5 Collagen synthesis

Cells were incubated with 5 μ Ci/ml [³H-2,3]proline (2.5 Ci/mmol) for the last two hours of culture. Cell layers were lysed by freeze thawing and extracted with 0.5% Triton X-100. Samples were precipitated with cold 10% trichloroacetic acid, and acid insoluble material was collected by centrifugation. Precipitates were extracted with acetone, dried, and rehydrated. [³H-2,3]proline incorporated into collagenase digestible protein (collagen) or noncollagen proteins was determined by differential sensitivity to bacterial collagenase free of nonspecific protease activity. Percent collagen synthesis was calculated by correcting for the 5.4-fold enrichment of proline content in collagen (Centrella et al., 1987; Centrella et al., 1995).

2.6 Alkaline phosphatase activity

Cell layers were lysed by freeze thawing and extracted with 0.5% Triton X-100. Enzyme specific activity was assessed in cell extracts by hydrolysis of *p*-nitrophenol phosphate, measured at 410 nm after 30 minutes of incubation at 37°C. Data are expressed as pmol of *p*-nitrophenol released per minute per μ g protein (Centrella et al., 1987; Centrella et al., 1995).

2.7 mRNA analysis

Total RNA was extracted with acid guanidine monothiocyanate, precipitated with isopropyl alcohol, dissolved in sterile water (Centrella et al., 1995), and purified further using the RNeasyTM Plus Mini Kit (Qiagen). RNA was reverse transcribed with the iScriptTM cDNA Synthesis Kit and products were amplified and quantified using specific primers (eurofins

mwg/operon) and the iQ™ SYBR® Green Supermix in a CFX96 Real Time polymerase chain reaction (RT-PCR) detection system (Bio-Rad Laboratories, Inc.). Alternately, IGF-I mRNA was determined by ribonuclease protection assay, where antisense ³²P-labeled RNA probes prepared from the cloned rat IGF-I gene and 18S rat rRNA were hybridized with total osteoblast RNA, digested with ribonuclease A and ribonuclease T1. Protected fragments were resolved on a 5% denaturing polyacrylamide gel and visualized by autoradiography as described previously (McCarthy et al., 2000b). To determine mRNA turnover, cells were treated with the RNA polymerase II inhibitor 5,5-dichloro-1-b-D-ribofuranosyl-1 H-benzimidazole (Sigma) at 50 μM (Shin et al., 1997) for 6, 24, or 48 hours, and mRNA levels were measured by RT-PCR as above.

2.8 Electrophoretic mobility shift assay (EMSA)

Overhangs on a double-stranded DNA probe (Life Technologies Inc.) containing the Runx and Smad binding sequences from the IGF-I gene promoter were filled with dNTPs and [α -³²P]dCTP using the Klenow fragment of DNA polymerase I. The sequence of the [α -³²P]dCTP probe and oligonucleotides containing the native and/or mutated sequences used for competition binding with the labeled probe are shown in table 1. Three μg of nuclear protein was pre-incubated with 2 μg of poly(dI:dC), without or with unlabeled specific or nonspecific competitor DNAs or antibody preparations, supplemented with 5 × 10⁴ cpm of probe (0.2 ng), and fractionated through a 5% nondenaturing polyacrylamide gel. Radioactive bands were visualized by autoradiography (McCarthy et al., 2000b).

2.9 Chromatin immunoprecipitation

Cells were formaldehyde cross-linked, collected by scraping, and detergent lysed, and chromatin was fragmented by sonication to approximately 700 bp. Samples were diluted and pre-cleared with Protein A Sepharose, incubated with nonimmune immunoglobulin IgG or anti-Runx2 antibody, and collected with Protein A Sepharose. They were digested sequentially with ribonuclease A and proteinase K, incubated overnight at 68 °C to reverse cross-linking, extracted with phenol and chloroform, and precipitated with ethanol. Equal aliquots of extracted DNA were amplified by PCR with primers corresponding to the sequences at nucleotides that encompass the Runx RE at nucleotides -1494 and -1488 in the rat IGF-I gene promoter. The specific PCR product was visualized by ethidium staining (Kim et al., 2006).

2.10 Statistical analysis

Differences were assessed by one way analysis of variance with *post hoc* analysis when comparing multiple treatments simultaneously, using SigmaStat software (Jandel Corporation), which defaults to Student's *t* test when assessing single treatments, from data derived from nine or more replicate samples and three or more independent cell preparations. mRNA levels were from three or more studies. A significant difference was assumed by a *P* value of <0.05.

3. RESULTS

3.1 Hypoxia induced changes in osteoblast activity

To establish that rat osteoblasts generate a typical hypoxic response, we measured the activity of endogenous HIF in cells transfected with a reporter plasmid driven by five HIF REs (Yun et al., 2005). Under ambient normoxia, HIF dependent gene expression was high, and it increased by 70–90% after 24 hours of exposure to hypoxia. HIF activity was also enhanced in cells treated with cobalt chloride to block the activity of prolylhydroxylase 2 and fully suppress native HIF proteolysis as a positive control, confirming that osteoblasts express a high level of HIF that is vulnerable to rapid turnover (Fig. 1A). In addition, we measured several general aspects of normal osteoblast activity in response to hypoxia. Whereas 24 hours of hypoxia or cobalt chloride significantly suppressed DNA synthesis rates (Fig. 1B), it produced little or no change in collagen or general protein synthesis (Fig. 1C), or alkaline phosphatase activity (Fig. 1D). We then examined a small panel of mRNAs which were chosen for their relatively constitutive expression, their association with hypoxia, with osteoblast differentiation, or their relationship to hormone dependent IGF-I gene synthesis by these cells. Within this group of mRNAs, which differed in basal expression by five orders of magnitude, thirteen were unchanged, three increased and seven decreased in response to short term exposure to either hypoxia or cobalt chloride (Fig. 1E). Of the three mRNAs that increased, all responded to both hypoxia and cobalt chloride, including the well known HIF-sensitive mRNA encoding vascular endothelial growth factor (VEGF) (Madri, 2009) (Fig. 1F). Of the seven mRNAs that decreased, several that are associated with differentiated osteoblasts were suppressed by cobalt chloride. Of these, however, only IGF-I and transcription factor Runx2 mRNAs were also suppressed by hypoxia (Fig. 1G). Despite the direct relationship that we previously reported between C/EBP δ and IGF-I expression in osteoblasts (Umayahara et al., 1997; Umayahara et al., 1999; McCarthy et al., 2000a; McCarthy et al., 2000b; Billiard et al., 2001), there was a clear dichotomy between C/EBP δ mRNA (which increased) and IGF-I mRNA (which decreased) with hypoxia (Fig. 1F and G). However, when we examined mRNA turnover rates, hypoxia failed to alter the relatively more rapid turnover of C/EBP δ or the relatively slower turnover of IGF-I mRNA in cells treated with an RNA polymerase II inhibitor to suppress new mRNA synthesis. Hypoxia also failed to alter the turnover of mRNAs encoding Runx2, C/EBP β , VEGF, or HIF1 α (supplemental Fig. 1). Thus hypoxia appeared to drive relatively restricted effects on osteoblasts.

3.2 Combined response to hypoxia and PGE₂

Given the ample evidence for the stimulatory effect of PGE₂ on IGF-I expression in osteoblasts (Umayahara et al., 1997; Umayahara et al., 1999; McCarthy et al., 2000a; McCarthy et al., 2000b; Billiard et al., 2001), we then determined the combined effects of hypoxia and PGE₂, which itself is induced in osteoblasts in response to hypoxia (Lee et al., 2010). PGE₂ alone significantly increased IGF-I mRNA, and hypoxia reduced its effect to control levels (Fig. 2A). Even so, when we examined the activity of the proximal IGF-I gene promoter, which contains a key C/EBP RE in exon 1 (Umayahara et al., 1997; Umayahara et al., 1999), the potent stimulatory effect of PGE₂ was retained in hypoxia treated osteoblasts (Fig. 2B). Similarly, C/EBP δ and C/EBP β mRNA levels (Fig. 2C), and transgenic gene

promoter activity driven by endogenous C/EBP proteins through C/EBP REs (Fig. 2D), were both induced by PGE₂ and maintained with hypoxia. Variations in C/EBP dependent gene expression are likely due in large part to C/EBP δ since its relative copy number is 40-fold higher in the basal state by comparison to C/EBP β (Fig. 1 E). By contrast, Runx2 mRNA levels (Fig. 2E) and transgenic gene promoter activity driven by endogenous Runx2 protein through Runx REs (Fig. 2F) were both suppressed by hypoxia in PGE₂ treated osteoblasts. The inhibitory effect of hypoxia was unlikely related to a decrease in the *trans*-activation potential of either C/EBP δ or Runx2 since the ability of transgenic GAL4 DBD-fused C/EBP δ (Ji, 2003) or GAL4 DBD-fused Runx2 (Chang et al., 1998) to activate gene expression through *cis*-acting GAL4 REs was maintained or enhanced in hypoxia or hypoxia plus PGE₂ treated cells (supplemental Fig. 2). In total, these findings suggest that loss of IGF-I mRNA occurred despite high C/EBP δ mRNA and functional protein levels, and related more closely to variations in endogenous Runx2 expression.

3.3 Combined response to hypoxia and glucocorticoid

Since the response to stress is also controlled by systemic agents like glucocorticoid (Quax et al., 2013), we measured IGF-I expression and relevant endogenous transcription factor activity after short term exposure to a physiological level of the glucocorticoid cortisol. As in previous studies, cortisol enhanced transgenic gene promoter driven by glucocorticoid receptor (GR) through GR REs (Chang et al., 1998; McCarthy, 2003) (Fig. 3A), C/EBP δ and C/EBP β mRNA expression (McCarthy et al., 2000b) (Fig. 3B), and transgenic gene promoter activity driven by endogenous C/EBPs (Fig. 3C), effects which were all maintained with hypoxia. Despite these increases in C/EBP expression and activity, the inhibitory effect of hypoxia on IGF-I mRNA expression remained in the presence of cortisol (Fig. 3D). Notably, IGF-I gene promoter activity also declined within this context (Fig. 3E), predicting a dominant influence by regulatory elements other than C/EBP. Consistent with our earlier studies revealing that cortisol reduces Runx2 dependent gene expression (Chang et al., 1998), the inhibitory effect of hypoxia on Runx2 expression (Fig. 3F) and endogenous Runx2 protein activity (Fig. 3G) was retained in cortisol treated osteoblasts.

3.4 Functional Runx RE in the IGF-I gene promoter

Based on these several observations, we reinspected the IGF-I gene promoter sequence and identified a consensus Runx RE within an upstream region at base pairs -1494 and -1488, flanked by two consensus REs for the transforming growth factor β (TGF- β) sensitive Smad transcription factors (Fig. 4A). Endogenous osteoblast-derived nuclear protein, from both control and PGE₂ treated osteoblasts, which associated with this sequence by EMSA, was depleted by Runx2 antibody (Fig. 4B). When we transgenically overexpressed Runx2, IGF-I mRNA levels increased (Fig. 4C), and Runx2 binding to this region was detected by chromatin immunoprecipitation (Fig. 4D). We also assessed the possible importance of the Smad binding sites within this region. When osteoblasts were stimulated with TGF- β , IGF-I gene promoter activity increased, and endogenous nuclear protein that associated with this sequence by EMSA was enhanced. With TGF- β treatment, nuclear protein binding was partially reduced by competition with oligonucleotide encoding the Smad consensus binding sequence, fully reduced by the Runx consensus binding sequence, not at all altered when the Runx binding sequence was mutated, and complex formation was highly depleted by anti-

Runx2 antibody (supplemental Fig. 3). Thus, the predominant and essential component of this complex is Runx2, which may synergize with Smads to drive IGF-I gene expression in response to TGF- β treatment. Notably, we previously reported that, analogous to C/EBP δ , Runx2 activity is sensitive to PKA activation in PGE₂ treated osteoblasts (McCarthy et al., 1995; McCarthy et al., 2000c). The importance of Runx2 for IGF-I expression also concurred with our earlier studies where we demonstrated that transgenic overexpression of Runx2 enhanced, and Runx2 silencing suppressed IGF-I gene promoter activity in PGE₂ treated osteoblasts (McCarthy et al., 2000c). Thus, our current studies now show that the stimulatory effect of PGE₂ and Runx2 on IGF-I extend beyond our previous evidence for their enhancing effects on C/EBP δ expression and activity. Moreover, when the Runx2 binding site within the full length IGF-I gene promoter was mutated, promoter dependent gene expression was reduced by approximately 60% in both uninduced and PGE₂ treated cells (Fig. 4E), corroborating the importance of endogenous Runx2 as a direct regulatory component of IGF-I expression in osteoblasts.

3.5 Overriding effect of hypoxia on Runx2 in stress hormone treated osteoblasts

With regard to the apparent disconnect between IGF-I mRNA and transgenic IGF-I gene promoter activity in response to hypoxia, we surmised that one or both of the C/EBP or Runx REs could have a dominant influence on innate gene expression or in response to the variations in the complex hormone environment *in vivo*. Therefore, we then measured the influence of hypoxia with the combination of PGE₂ and cortisol. In agreement with evidence that cortisol induces C/EBP δ and C/EBP β expression and that PGE₂ activates endogenous C/EBP dependent IGF-I expression (McCarthy et al., 2000b), IGF-I gene promoter activity increased synergistically in response to both agents. Even so, in the context of both agents, the inhibitory effect of hypoxia on IGF-I gene promoter activity was revealed (Fig. 4F), and this occurred in parallel with a persistent decrease in endogenous Runx2 dependent gene expression (Fig. 4G). Therefore, the only element that corresponded consistently with a decrease in IGF-I expression was a concomitant decrease in endogenous Runx2.

3.6 Stress hormone dependent control of HIF activity

By mRNA analysis, HIF1 α was expressed at a 6-fold higher level by comparison to HIF2 α (Fig. 1E). This difference may relate in part to an overall greater potency of the HIF1 α gene promoter and apparent silencer elements upstream of the 0.8 kb region in the HIF2 α gene promoter when they are expressed in differentiated osteoblasts (supplemental Fig. 4). Concerning HIF activity, the response to hypoxia and PGE₂ was relatively gene restricted inasmuch as neither HIF1 α nor HIF2 α mRNA levels were significantly modulated by these agents (Fig. 5A). By contrast to the other promoters that we tested, however, the stimulatory effect of hypoxia on endogenous HIF dependent transcriptional activity was synergistically enhanced by PGE₂ (Fig. 5B). In addition to its inhibitory effect on Runx2, cortisol suppressed HIF1 α mRNA levels in hypoxia treated osteoblasts (Fig. 5C), whereas it increased the level of the lower abundant HIF2 α mRNA. Even so, cortisol reduced overall endogenous HIF transcriptional activity (Fig. 5D) and suppressed the synergistic effect of PGE₂ (left panel, Fig. 5E). A reduction in endogenous HIF activity in response to cortisol may relate not only to a decrease in HIF1 α mRNA levels, but also to the ability of cortisol to

increase C/EBP δ expression (Fig. 3B), since transgenic C/EBP δ expression completely reprised its inhibitory effects (right panel, Fig. 5E).

3.7 Persistent effects by longer term hypoxia

Lastly, the combined effects of these regulatory agents and events were maintained and progressed even further with prolonged, 144 hours, of exposure to hypoxia. That is, IGF-I gene promoter activity (Fig 6A) and endogenous Runx2 activity (Fig. 6B) were both potently reduced by prolonged hypoxia in cortisol treated and PGE₂ induced osteoblasts, and endogenous HIF activity in both control and hypoxia induced cells was potently suppressed by cortisol (Fig. 6C).

4. DISCUSSION

Our current studies provide novel evidence for a previously unrecognized inhibitory effect by hypoxia on IGF-I expression by osteoblasts, and reveal that this can occur in part by limiting the influence of an upstream Runx response element in the IGF-I gene promoter. Indeed, earlier studies predicted that Runx2 levels in osteoblasts were diminished by hypoxia (Park et al., 2002; Salim et al., 2004; Yang et al., 2011; Nicolaije et al., 2012), which we here extend to a Runx2 sensitive gene, IGF-I, that is essential for bone integrity (McCarthy et al., 1989; McCarthy and Centrella, 2001). We further show that the regulatory effects of hypoxia are compounded by the local and systemic stress hormones PGE₂ and cortisol, which all converge on Runx2 and IGF-I. The literature is replete with evidence that hypoxia is linked to components of the IGF system (Smith, 2004; Kietzmann et al., 2006; Tchirikov et al., 2006; Lee et al., 2013; Radom-Aizik et al., 2013), but there has been little attention to this in bone cells except for a preliminary study suggesting that IGF-II mRNA increases and IGF-I mRNA remains unchanged in response to 48 hours of exposure to 5% oxygen in newborn rat derived cells (Steinbrech et al., 2000). However, these initial studies may have been performed at a stage of cell differentiation where Runx2 levels were insufficient to drive high levels of IGF-I expression, or that a reduction of oxygen to the 5% level is insufficient to deplete Runx2 (Salim et al., 2004), and account for this disparity with our current results. Further supporting a link between IGF-I and hypoxia, IGF-I administration has been heavily addressed as a possible first line defense tool to counteract hypoxic damage in many tissues, consistent with its loss under limited oxygen conditions (Guan et al., 2003; Smith, 2003; Chen and Smith, 2007; Mantagos et al., 2009). In this regard, IGF-I is well known to induce HIF expression by many cells including osteoblasts (Mimeault and Batra,; Akeno et al., 2002; Fukuda et al., 2002; Poulaki et al., 2004; Tang et al., 2010; Sinha et al., 2011; Yu et al., 2012), where it can drive VEGF expression and increase local blood vessel growth and enhance tissue repair.

We were surprised by the lack of an immediate effect by hypoxic stress on endogenous protein synthesis (Koritzinsky et al., 2006; Koumenis and Wouters, 2006) as well as other aspects of osteoblast differentiation, including type I collagen expression, alkaline phosphatase activity, or the expression of a panel of mRNAs well associated with these cells (Salim et al., 2004; Utting et al., 2006; Hirao et al., 2007; Yang et al.,; Nicolaije et al., 2012). Several of these mRNAs were nonetheless limited by cobalt chloride treatment. Undeniably, effects previously seen in other cell models may occur in osteoblast-destined

mesenchymal cells at earlier stages of differentiation, or with progressive exposure to hypoxia. Even so, some studies predict a recovery over time after cells adjust to and establish a new, lower, set point for oxygen sensing, which itself may be determined by differences in the relative levels of prolyl-hydroxylase or HIF α isoforms (Khanna et al., 2006; Stiehl et al., 2006; Yuan et al., 2013). Still, in addition to its effect on HIF turnover, cobalt chloride could alter the levels of other transcriptional regulators that enhance or suppress the expression of still other gene products, which are not yet been as well defined.

Although IGF-I and Runx2 expression are clearly associated with osteoblasts and bone formation, to our knowledge our current studies provide the first evidence that Runx2 contributes to the process of IGF-I expression in these cells. The complexity of the IGF-I gene promoter, in particular with regard to the upstream Runx RE that we identify here and the critical downstream C/EBP RE that we previously defined (Umayahara et al., 1997; Umayahara et al., 1999), suggested the possibility of a stratified response to multiple regulatory signals. Notably, hypoxia suppressed the potent stimulatory effect of PGE₂ on IGF-I mRNA expression and enforced the inhibitory effect of glucocorticoid. Similar to the C/EBPs (Centrella et al., 2004), the stimulatory effect of Runx2 on IGF-I expression is regulated by PGE₂, and this may also contribute to the loss of IGF-I expression that occurs in response to hypoxia. Furthermore, as in our earlier studies, a major response to glucocorticoid was a reduction in Runx2 dependent gene expression (Chang et al., 1998), predicting that loss of IGF-I when Runx2 levels decline is yet another mechanism for bone loss with glucocorticoid excess. Analogous effects may occur in other tissues where Runx2 or other Runx isoforms exist, and account for glucocorticoid induced changes in cell function, including cartilage, immunological, breast, neurological, and gastric tissues where members of this transcription factor family have potent supportive and suppressive roles (Cohen, 2009; Zagami et al., 2009; Blyth et al., 2010; Naito et al., 2011; Ching and Frenkel, 2013; Chuang et al., 2013).

Earlier studies in osteoblasts and other cell types showed potent interactions between hypoxia and PGE₂, often through an increase in endogenous PG synthesizing enzymes and PG production (Lee et al., 2007; Lee et al., 2010). Unlike results that occur in several other cells types (Ji et al., 2010; Fernandez-Martinez et al., 2012a; Fernandez-Martinez et al., 2012b), however, we found no increase in HIF α subunit expression by PGE₂, although we did observe potent synergy between PGE₂ and hypoxia on HIF activity. This might occur through a PGE₂ dependent increase in HIF α activation that can occur independently of HIF α synthesis, as we reported for C/EBP δ and Runx2 (McCarthy et al., 2000c; Ji, 2003), but to our knowledge HIF α activation has not been previously addressed in this context. Alternately, this could depend on an increase in HIF α stability or direct physical interactions between HIF α subunits and other basal or induced transcriptions factors, which we have not yet resolved. Regardless, we found a far greater abundance of HIF1 α mRNA expression relative to HIF2 α , perhaps related to a higher basal HIF1 α gene promoter activity and the presence of an upstream suppressor element in the HIF2 α gene promoter, which predicts a greater role for HIF1 α versus HIF2 α in osteoblasts. This might account for variations in basal hypoxia dependent gene expression, as found in other cells where ratio of HIF1 α to HIF2 α drives a change in the oxygen sensing set point (Yuan et al., 2013). Furthermore, high HIF1 α levels have often been associated with a decrease in the proliferation of some

non-oncogenic cells (Goda et al., 2003; Zhu et al., 2005; DeBerardinis et al., 2008; Higgins et al., 2008; Millman et al., 2009), which is also consistent with our results in osteoblasts. By contrast, glucocorticoid reduced HIF activity, which has been seen in some but not all cell types (Kodama et al., 2003; Hata et al., 2008; Wagner et al., 2008; Gaber et al., 2011), and may depend in part on a physical complex that forms between hormone activated GR and HIF1 α (Sun et al., 2010). Based on our current and previous studies, this may also result from an increase in endogenous C/EBP expression in glucocorticoid treated osteoblasts, inasmuch as over expression of C/EBP δ also suppressed HIF activity. In addition, variations in HIF activity could derive from a physical complex formed by HIF1 α with C/EBP δ , similar to its interaction with C/EBP α , which is inhibitory in some contexts and stimulatory in others (Jiang et al., 2005; Janardhan, 2008; Zhao et al., 2011). Again, hypoxic stress has been linked to a rapid decrease in protein synthesis through phosphorylation of initiation factor eIF2 (Koritzinsky et al., 2006; Koumenis and Wouters, 2006), an effect that we did not observe. This may depend on the relatively high levels of HIF1 α to HIF2 α , which could establish a different set point for hypoxic sensing in differentiated osteoblasts (Yuan et al., 2013), and thus allow variations in response that relate to cell phenotype, physiological context, duration of exposure, and hormonal environment.

In summary, we report here that hypoxia reduces endogenous IGF-I gene expression by osteoblasts and suppresses the potent stimulatory effect of PGE₂ on this process. This correlates consistently with a decrease in Runx2, which binds to and enhances IGF-I expression through an upstream element in the IGF-I gene promoter, independent of the previously defined downstream C/EBP element in exon 1. The inhibitory influence of hypoxia is enforced by glucocorticoid, which also diminishes Runx2 levels and activity. Thus, we have defined new evidence for control of IGF-I expression by osteoblasts, a previously unrecognized transcriptional regulatory element in the IGF-I gene promoter, and stratified interactions among hypoxic stress and the influence of local and systemic stress hormones that converge on IGF-I through effects on the obligate osteogenic regulator Runx2. A model for these findings is shown in Fig. 7. How these events ultimately control variations in gene expression by cells during the process of osteoblast differentiation, or within various compartments in bone where differences in oxygen tension or hormone sensitivity may occur will require further studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

cAMP	cyclic AMP
C/EBP	CCAAT enhancer binding protein
DBD	DNA binding domain
EMSA	electrophoretic mobility shift assay
GR	glucocorticoid receptor
HIF	hypoxia induced transcription factor
IGF-I	insulin-like growth factor I
PG	prostaglandin
PKA	protein kinase A
RE	response element
RT-PCR	real time polymerase chain reaction
Runx	<i>runt</i> homology domain transcription factor
TGF-β	transforming growth factor β
VEGF	vascular endothelial growth factor

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Highlights

1. Hypoxia suppresses osteoblast IGF-I and Runx2 expression
2. Hypoxia counteracts the stimulatory effects of PGE₂
3. Glucocorticoids enforce hypoxic inhibition
4. The IGF-I gene promoter contains a functional Runx2 response element
5. IGF-I expression is stratified by hypoxia and stress hormones through Runx2

Fig. 1. Hypoxia induced changes in osteoblast activity

Osteoblasts were cultured for 24 hours under normoxia of 22% oxygen (norm), hypoxia of 1% oxygen (hyp) or supplemented with 100 μ M cobalt chloride (cob) under normoxia to suppress prolyl hydroxylase and suppress endogenous HIF turnover. In panel A, the cells were transfected for 24 hours with a luciferase reporter plasmid driven by 5 HIF REs before treatments, and luciferase activity was measured. In panel B, DNA synthesis rates were measured by [³H]thymidine incorporation during the last 2 hours of treatment. In panel C, collagen and general protein synthesis rates were measured by [³H]proline incorporation during the last 2 hours of treatment. Cell extracts were digested with collagenase to determine the total amount of collagen synthesis, and percent collagen synthesis was calculated by the 5.5-fold relative enrichment of proline in collagen versus noncollagen proteins. In panel D, alkaline phosphatase was measured by substrate conversion and spectrophotometry. In panels E, F and G, mRNA levels were measured by real time PCR. Copy numbers for individual mRNAs, relative to each other and standardized by 18s rRNA recovery are shown in panel E, and mRNA levels relative to normoxia are shown in panels F and G. Hypoxia and cobalt each significantly enhanced HIF activity in panel A and reduced DNA synthesis in panel B. No significant differences were present on protein synthesis or alkaline phosphatase activity in panels C and D. In panel F, hypoxia and cobalt chloride each significantly enhanced C/EBPδ, CXCR4, and VEGF mRNA expression. In panel G, cobalt chloride significantly suppressed all seven mRNAs shown, whereas hypoxia significantly suppressed only IGF-I and Runx2.

Fig. 2. Combined response to hypoxia and PGE₂

Osteoblasts were cultured under normoxic or hypoxic conditions for 24 hours as in Fig. 1, and then treated for 6 hours with control medium or 1 μ M PGE₂ to induce IGF-I expression. In panels A, C and E, mRNA levels were measured by real time PCR relative to normoxia and standardized by 18s rRNA recovery. In panels B, D, and E, cells were transfected for 24 hours with luciferase reporter constructs driven by the rat IGF-I gene promoter (panel B), by 4 authentic C/EBP REs (panel D), or by 2 authentic Runx REs (panel F) before treatments. IGF-I mRNA was significantly enhanced by PGE₂ alone, whereas C/EBP δ and C/EBP β mRNAs were significantly enhanced by PGE₂ when osteoblasts were cultured under normoxic or hypoxic conditions. By contrast, Runx2 mRNAs were significantly reduced by hypoxia in both control and PGE₂ stimulated cells. PGE₂ significantly enhanced luciferase reporter constructs driven by the IGF-I promoter and by C/EBP REs when osteoblasts were cultured under normoxic or hypoxic conditions, whereas hypoxia significantly reduced reporter constructs driven by Runx REs in both control and PGE₂ treated osteoblasts.

Fig. 3. Combined response to hypoxia and glucocorticoid

Osteoblasts were cultured under normoxic or hypoxic conditions for 24 hours as in Fig. 1, in the absence (control) or presence of 100 nM cortisol to induce C/EBP δ and C/EBP β expression. Cells were transfected for 24 hours with luciferase reporter constructs driven by the MMTV promoter (GR RE) that associates with the hormone activated GR (panel A), by 4 authentic C/EBP REs (panel C), by the rat IGF-I gene promoter (panel E), or by 2 authentic Runx REs (panel G) before treatments. In panels B, D, and F, mRNA levels were measured by real time PCR relative to normoxia and standardized by 18s rRNA recovery. Cortisol significantly enhanced GR dependent and C/EBP dependent gene promoter activity in panels A and C, but significantly reduced IGF gene promoter and Runx dependent gene promoter activity in panels E and G. Cortisol also significantly enhanced C/EBP δ and C/EBP β mRNA levels in panel B, but significantly reduced IGF-I mRNA levels in panel D.

Fig. 4. Functional Runx RE in the IGF-I gene promoter

Osteoblasts were cultured under normoxic or hypoxic conditions or treated with cortisol and/or stimulated with PGE₂ as in Figs. 1–3, as indicated. Panel A shows the locations of the Runx and Smad binding elements in the IGF-I gene promoter by reference to the previously defined C/EBP binding element in exon 1. Panel B shows nuclear protein binding to the Runx/Smad binding sequence by EMSA from control (O) and PGE₂ stimulated osteoblasts in extracts supplemented with control antibody (IgG) and anti-Runx2 antibody (αRunx2). Panel C shows IGF-I mRNA (above) and 18S rRNA (below) in extracts from osteoblasts transfected with empty vector or expression vector encoding mouse Runx2, as determined by ribonuclease protection (RP) assay. Panel D shows chromatin precipitation of Runx2 in combination with DNA from osteoblasts collected with nonspecific IgG or αRunx2, using primers that define the region containing the Runx/Smad binding sequence shown in panel A. Plasmid DNA containing the same sequence was used as a positive control. Cells were transfected with luciferase reporter constructs driven by the native rat IGF-I gene promoter, or with the IGF-I gene promoter containing a mutated Runx2 binding site (see table 1) in panels E and F, or by 2 authentic Runx REs in panel G, before the treatments indicated. IGF-I gene promoter activity was significantly reduced by mutation of the Runx2 binding site in both control and PGE₂ stimulated cells in panel E, and by hypoxia in cortisol treated and PGE₂ stimulated cells in panel F. Cortisol and hypoxia, individually and together significantly reduced Runx dependent gene promoter activity in control and PGE₂ stimulated osteoblasts in panel G.

Fig. 5. Stress hormone dependent control of HIF activity

Osteoblasts were cultured under normoxic or hypoxic conditions or treated with cortisol and/or stimulated with PGE₂ as in Figs. 1–3, as indicated. In panels A and C, mRNA levels were measured by real time PCR relative to normoxia and standardized by 18s rRNA recovery. In panels B, D, and E, cells were transfected with luciferase reporter constructs driven by five authentic HIF REs before the treatments indicated. Cultures on the right side of panel E were co-transfected with empty vector or an expression plasmid encoding rat C/EBP δ . Hypoxia had no effect on HIF1 α or HIF2 α mRNA levels in control or PGE₂ treated osteoblasts in panel A, whereas cortisol significantly reduced HIF1 α mRNA levels in hypoxia treated cells and significantly enhanced HIF2 α mRNA levels in normoxic and hypoxia treated cells in panel C. Hypoxia and PGE₂ individually and together significantly enhanced HIF dependent gene promoter activity in panel B, whereas cortisol significantly reduced the stimulatory effect of hypoxia in panel D and the co-stimulatory effects of hypoxia and PGE₂ in panel E. In panel E, the significant inhibitory effect of cortisol, as shown on the left, was reprised by transgenic C/EBP δ expression, as shown on the right.

Fig. 6. Persistent effects by longer term hypoxia

Osteoblasts were cultured under normoxic or hypoxic conditions for 144 hours without or with 100 nM cortisol, and then treated for 6 hours with control medium or 1 μ M PGE₂ to induce IGF-I expression. Cells were transfected for 24 hours with luciferase reporter constructs driven by the rat IGF-I gene promoter (panel A), by 2 authentic Runx REs (panel B), or by 5 authentic HIF REs (panel C) before treatments. In panel A, hypoxia significantly reduced the co-stimulatory effects of cortisol and PGE₂ on IGF-I gene promoter activity. In panel B, hypoxia reduced Runx dependent gene promoter activity in cortisol, PGE₂, or cortisol plus PGE₂ treated cells. In panel C, cortisol significantly reduced the stimulatory effect of hypoxia and the co-stimulatory effects of hypoxia and PGE₂.

Fig. 7. Model

Our present studies lead us to develop a model indicating that hypoxia reduces endogenous IGF-I gene expression by osteoblasts and suppresses the stimulatory effect of PGE₂ on this process. This correlates with a decrease in Runx2, which binds to and enhances IGF-I expression through an upstream element in the IGF-I gene promoter, independent of the previously defined downstream C/EBP element in exon 1. The inhibitory influence of hypoxia is enforced by glucocorticoid, which also diminishes Runx2 levels and activity. Thus, we have defined stratified interactions among hypoxic stress and the influence of local and systemic stress hormones that converge on IGF-I through effects on the obligate osteogenic regulator Runx2.

Table 1

Oligomers used for nuclear protein binding by EMSA *

Protein	Sequence, 5' → 3'
Runx/Smad (<i>igf1</i>)	TGTGAGGTGTTGACCACACAGAGGATT
Runx/SmadμR (<i>igf1</i>)	TGTGAGGTGTTG <u>Att</u> ACACAGAGGATT
Smad CS	CGGAGTATGTCTAGACTGACAATGTAC
Runx CS	GGTTGGTTA <u>ACCAC</u> AGGTTGG

* Transcription factor binding sequences (underlined) were obtained by the MatInspector program from Genomatix (<http://www.genomatix.de>). Oligonucleotides designated by the addition of (*igf1*) are native or mutated sequences derived from the IGF-I gene promoter region. Nucleotide substitutions within a mutated Runx binding site (μR) is indicated by lower case letters. Other oligonucleotides are unrelated to the IGF-I gene promoter sequence but contain either Smad or Runx consensus binding sequences (CS).