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Hypoxia increases Annexin A2 expression in osteoblastic cells via VEGF and ERK

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Abstract

Vascular endothelial growth factor (VEGF) stimulated angiogenesis is critical for endochondral ossification that occurs during bone development, and bone repair. Under these circumstances, VEGF production appears to be driven by low oxygen tension, under the control of the hypoxia inducible factor- α family of transcription factors (HIF- α). Annexin 2 (AnxA2) a calcium dependant phospholipid binding protein has been implicated in VEGF-mediated retinal neovascularization and is upregulated by VEGF in choroid retinal endothelial cells. AnxA2 is also expressed in cells of the osteoblast lineage and chondrocytes and may play a role in matrix mineralization. In this paper we examined the effects of hypoxia (1% O₂) and VEGF on the expression of AnxA2 in osteoblastic MC3T3-E1 cells. Hypoxia, desferrioxamine (hypoxia mimetic) and recombinant VEGF all increased AnxA2 mRNA and protein levels in osteoblastic cells. The hypoxia-induced increase in AnxA2 was inhibited by a blocking antibody to VEGF-R1, however, VEGF₁₂₀, a VEGF-R1 agonist demonstrated no influence upon *Anxa2* expression. This suggests that VEGF induction of Annexin A2 is not mediated *via* VEGF-R1 agonism alone, but by VEGF-R1 and Neuropilin-1 or Neuropilin-2 heterodimers. Additionally we demonstrated that VEGF-stimulated changes in AnxA2 expression via a pathway involving Src and MEK kinase. These data demonstrate that AnxA2 expression in osteoblasts is under the control of VEGF, which may have implications for both angiogenesis and bone mineralization under low oxygen conditions.

Keywords

Osteoblast; Hypoxia; Annexin A2; VEGF; ERK; Src

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Introduction

Longitudinal bone growth occurs through endochondral bone formation. Within the epiphyseal growth plate, a region of resting chondrocytes undergoes proliferation, followed by differentiation into hypertrophic chondrocytes. Apoptosis of hypertrophic chondrocytes is followed by blood vessel invasion, resorption of calcified cartilage, and its replacement with bone [1]. Vascular endothelial growth factor (VEGF), produced by hypertrophic chondrocytes, is essential for growth-plate angiogenesis. Gerber *et al.* demonstrated that sequestration of VEGF with a soluble chimeric protein enlarged the growth plate *via* reductions in the apoptosis of hypertrophic chondrocytes, blocked blood vessel invasion, and ultimately decreased longitudinal bone growth [2]. Expression of VEGF in hypertrophic chondrocytes, and other cell phenotypes, increases under conditions of reduced pericellular oxygen tension (hypoxia) that is driven by the hypoxia-inducible factor- α (HIF- α) family of transcription factors [3].

The annexins are a group of structurally related Ca^{2+} -binding proteins that bind to membrane phospholipids in a calcium-dependent manner [4, 5]. They perform various roles within and outside of a cell, as isoforms have been implicated in intracellular Ca^{2+} homeostasis, vesicle aggregation, cytoskeleton binding, and the establishment and maintenance of microdomains within the plasmalemma [5]. We have previously demonstrated that Annexin A5 (AnxA5) is central to osteoblast mechanotransduction, as chemical or antibody inhibition of AnxA5 significantly decreased fluid shear stress-induced Ca^{2+} signaling and gene expression [6]. Another annexin isoform, annexin A2 (AnxA2), is expressed in cells of the osteoblast lineage including rat calvarial osteoblasts [7], human long bone osteoblasts [8], mouse MC3T3-E1 [8-10] rat UMR-106 cells [9], rat ROS 24/1 cells and human osteosarcoma Saos-2 and SaOSLM2 cells [11, 12]. Anxa2 is also expressed in mesenchymal stem cells that have osteogenic potential, including those derived from human bone marrow [13, 14] and human umbilical cord [15]. The exact role of annexin 2 in osteoblast biology is unknown, although evidence suggests that it plays a critical role in the process of matrix mineralization in hypertrophic chondrocytes, vascular smooth muscle cells, and osteoblastic cells [10, 12, 16, 17].

Annexin A2 also plays a role in angiogenesis and neovascularization. Firstly, Annexin A2 is a receptor for the angiogenic-related proteins angiostatin and tissue plasminogen activator [18-22]. Secondly, Annexin A2 is also involved in VEGF-mediated neovascularization. Zhao *et al.* reported that *AnxA2* mRNA and Annexin A2 protein were increased in a murine model of ischemic retinopathy through a VEGF/VEGFR2/PKC β pathway [23]. Thus, we sought whether Annexin A2 is similarly controlled by hypoxia and VEGF in osteoblastic cells.

Materials and Methods

Cell culture

MC3T3-E1 osteoblastic cells were kindly provided by Dr. Norman Karin (Pacific Northwest National Laboratory, Richland, WA). Cells were maintained under 5% CO_2 -95% ambient air in humidified incubators. Medium was Minimal Essential Medium, Alpha modification,

(Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin. Cells were routinely sub-cultured with 0.5% trypsin when 80-90% confluent. For studies described within, cells were sub-cultured and seeded at a density of 5,000/cm²; studies were performed on the following day.

Preparation of RNA from murine femora

The femora were removed from 12 - 13 week-old male mice and cleaned of surrounding soft tissue; epiphyses were cut off with scissors, and the marrow was flushed out with PBS. The remaining femora were dropped into a pestle filled with liquid nitrogen and ground to dust using a mortar, followed by a standard Trizol extraction.

Chemicals

Desferioxamine was purchased from Sigma. Recombinant murine VEGF₁₆₄, VEGF₁₂₀, and a VEGF-neutralizing antibody were purchased from Peprotech. Inhibitors for MEK1/2 (U0126), Src (PP2), VEGF-R1 (CPO-B11) were purchased from EMD Biosciences.

Hypoxic culture

Cells were transferred into humidified incubators at 37°C with 5% CO₂ and oxygen tension reduced to 10%, 5%, or 1% using supplemental N₂ (HERAcell 150, Kendro, USA), or maintained in a standard humidified incubator for normoxia (21% oxygen tension). Media was pre-conditioned in each oxygen tension for 24 hours.

Quantitative PCR

Cells were washed with PBS and total RNA was collected using RNeasy Mini kit (Qiagen). 0.5 – 1 microgram of total RNA was reverse-transcribed with QuantiTect Reverse Transcription Kit (Qiagen), which includes a genomic DNA elimination step. qPCR was performed using QuantiFast Probe PCR Kit (Qiagen) on a Mastercycler[®] realplex2 (Eppendorf). Proprietary primer and probe sets for *Vegf*, *AnxA2*, *Nrp1*, *Nrp2*, *VegfR1*, *VegfR2*, *VegfR3*, and *TubA* were purchased from Applied Biosystems. Amplification conditions were 95°C for 3 minutes, followed by 40 cycles at 95°C for 3 seconds and 60°C for 30 seconds. Quantitative PCR results were first normalized to loading control (*TubA*) transcript level to yield C_t , then normalized to control conditions (*e.g.*, normoxia at the same time point) to generate C_t . Relative or fold change in expression was subsequently calculated using the formula 2^{-C_t} or 2^{-C_t} , respectively [24].

Western immunoblotting

Cells were briefly washed with PBS and whole cell protein lysates were collected in 0.1% Triton X-100, 10mM Tris, pH 8, 1mM EDTA, supplemented with a protease and phosphatase inhibitor cocktail (Pierce-ThermoFisher). Samples were resolved in 10% Bis-Tris gels (Invitrogen), transferred onto 0.2µm nitrocellulose membranes, and blocked in non-fat milk in Tris-buffered saline supplemented with 0.1% Tween-20. Proteins were detected with antibodies directed against Annexin A2 (1:1000), phosphorylated (Tyr204; 1:250) or non-phosphorylated (1:1000) ERK1/2 (both from Santa Cruz Biotechnology), phosphorylated (Tyr416; 1:1000) or non-phosphorylated (1:1000) Src, β-actin (1:2000,

Abcam), or α -tubulin (1:1000, Cell Signaling). We have previously demonstrated that hypoxia does not influence α -tubulin or β -actin gene or protein expression [25]).

VEGF ELISA

Cell culture medium was removed, and replaced with fresh media, after which cultures were placed into incubators at 21% O₂ or 1% O₂. Media was collected 6, 24, or 48 hours later and frozen at -20C until analysis; corresponding whole-cell protein lysates were also collected, for normalization of VEGF release. The mouse VEGF Quantikine ELISA kit (R&D Systems), which recognizes murine VEGF₁₆₄ and VEGF₁₂₀, was used according to the manufacturer's instructions.

Statistical analysis

Each data set was acquired a minimum of three times, in duplicate. Data were analyzed by Kruskal-Wallis or ANOVA followed by Dunnet or Tukey *post-hoc* tests where appropriate.

Results

Hypoxia increases Annexin A2 expression

The influence of reduced atmospheric oxygen tensions upon levels of *AnxA2* transcript was examined in MC3T3-E1 osteoblasts after 3, 6, or 24 hours of culture; expression of *Vegf*, a classic hypoxia-induced gene, was also monitored. Levels of *Vegf* transcript significantly increased after 3 or 24 hours of hypoxic culture compared to normoxic-matched controls (Figure 1A). Likewise, hypoxia also exerted a stimulatory effect upon *AnxA2* transcript (Figure 1A), although hypoxic culture was not as stimulatory for *AnxA2* as for *Vegf*. Further, the induction of *AnxA2* proceeded more slowly than for *Vegf*, as maximal hypoxia-induced expression of *AnxA2* occurred after 24 hours of culture. Changes in mRNA expression were confirmed at the level of protein by Western immunoblotting, wherein hypoxic culture for 24 hours demonstrated significant increases in annexin A2 levels (Figures 1B and 1C).

Reductions in pericellular O₂ tension inhibit the ability of the von Hippel-Landau (VHL) E3 ligase to ubiquitinate the hypoxia-inducible factor- α (HIF- α) family of transcription factors, which thereby enables the transcription of HIF-responsive genes [26, 27]. Because hydroxylation requires iron as a co-factor, it is possible to stabilize HIF- α isoforms, and thereby induce HIF-driven transcription, by treating cells with an iron chelator [28]. As shown in Figure 2A and 2B, MC3T3-E1 osteoblastic cells cultured at 21% O₂ in the presence of 50 or 100 μ M desferioxamine (DFO) for 24 hours reveal increased Annexin A2 protein expression compared to DFO-free controls. These data suggest that stabilization of HIF- α isoforms is responsible for hypoxic induction of Annexin A2 mRNA and protein expression, although it does not exclude the possibility that reactive oxygen species contribute to HIF-1 α stabilization.

VEGF₁₆₄ increases Annexin A2 levels

Vascular endothelial growth factor (VEGF) expression increases under hypoxic conditions [29, 30]. We demonstrated in Figure 1A that *Vegf* transcript increased under 1% O₂ versus 21% O₂-matched cultures; these data were confirmed at the protein level with ELISAs for

VEGF₁₆₄ and VEGF₁₂₀. We observed robust and sustained release of these VEGF isoforms under conditions of 1% O₂ culture after 24 or 48 hours compared to 21% O₂ tension- and time-matched controls (Figure 3A).

Epithelial cells have been shown to increase *AnxA2* in response to VEGF [23], and we observed that addition of a VEGF-inhibiting antibody attenuated 1% O₂-induced *AnxA2* mRNA expression (Figure 3B). To further examine whether hypoxia influenced annexin A2 expression *via* VEGF, MC3T3-E1 osteoblasts were cultured with increasing concentrations of VEGF₁₆₄ at 21% O₂ for 6 or 24 hours. We observed significant increases in *AnxA2* expression in response to 10–100 ng/mL VEGF₁₆₄ after 6 hours (Figure 3C). These results were confirmed by Western immunoblotting, wherein 24hr culture with 25ng/mL VEGF₁₆₄ significantly increased Annexin A2 expression compared to vehicle controls (Figures 3D and 3E).

VEGF signals through VEGFR1 to induce Annexin A2 expression

Quantitative PCR revealed that MC3T3-E1, clone 14, osteoblastic cells express transcript for the VEGF receptors *VegfR1*, *Nrp-1*, and *Nrp-2*; no expression was observed for *VegfR2* or *VegfR3*, although *VegfR2* transcript was observed in RNA prepared from murine femora (Figure 4A). Inhibition of VEGFR1 with CBO-P11 (10μM) [31, 32] attenuated increases in Annexin A2 protein expression under hypoxia (Figure 4B), indicating the requirement of this receptor in hypoxia-driven Annexin A2 expression. Interestingly, the specific VEGF-R1 and VEGFR2 agonist, VEGF120 demonstrated no influence upon *AnxA2* expression, in contrast to VEGF₁₆₄ (Figure 4C). which suggests that VEGFR1 agonism alone is not sufficient for VEGF induction of Annexin A2. It is likely that this response is mediated *via* VEGF-R1 and Neuropilin-1 or Neuropilin-2 heterodimers.

VEGF-induced Annexin A2 expression involves Src and ERK1/2

Binding of VEGF to its receptors activates a variety of intracellular signaling pathways, including PI3K/Akt, Src, MAPK, and PLC (for review, see Olsson *et al.* [33]). Hypoxia has been shown to activate many of these same cascades [34–36]. Addition of VEGF₁₆₄ (25ng/mL) to cells cultured at 21% oxygen increased ERK1/2 phosphorylation relative to vehicle controls (Figures 5A and 5B) and demonstrated a trend for increased Src phosphorylation (data not shown). Maximal ERK1/2 phosphorylation was observed between 15–60 minutes after VEGF₁₆₄ addition, and began to decrease after 120 minutes. To determine whether VEGF₁₆₄-induced Src or ERK1/2 phosphorylation was required for changes in Annexin A2, MC3T3-E1 cells were pre-treated with the Src inhibitor PP2 (10μM) or the MEK inhibitor U0126 (10μM) for 30 minutes prior to, and during, VEGF₁₆₄ (25ng/mL) culture. The presence of PP2 significantly attenuated ERK1/2 phosphorylation in response to VEGF₁₆₄ (Figure 5C), indicating that VEGF signals through ERK1/2 *via* Src. Concomitantly, inhibition of either Src or MEK prevented VEGF₁₆₄ induction of *AnxA2* expression, indicating the requirement of these kinases in this process.

Stabilization of HIF-1α and *AnxA2* expression at a range of oxygen tensions

The oxygen tension to which cells osteoblastic cells ranges from most *in vitro* culture at 21% oxygen, approximately 5% within intact bone [37–40], to 0.8 – 3% during fracture

repair [41, 42]. To that end, we examined HIF-1 α stabilization (Figure 6A) and *AnxA2* transcript expression (Figure 6B) at oxygen tensions of 21%, 10%, 5%, and 1%. After 3 hours, we observed minor increases in HIF-1 α levels at 10% or 5% oxygen tension (50-80%) compared to 21% control, while 1% oxygen tension was increased nearly 400%. Similarly, *AnxA2* was slightly, but not significantly, increased (by 20%) at 5% oxygen tension, whereas it was significantly increased by 80% when cultured at 1% oxygen tension was after 24 hour culture.

Discussion

Reductions in pericellular oxygen promote adaptive changes in gene expression, primarily through stabilization of the HIF- α transcription factors; hypoxia target genes include those related to neoangiogenesis or vasculogenesis (VEGF and erythropoietin) and glycolysis (pyruvate kinase and GAPDH) [43]. While there exists differential and/or contradictory effects of reduced pericellular oxygen tension upon the various cell types present within bone, the functional link between angiogenesis and osteogenesis is firmly established. Inhibiting VEGF with a soluble receptor prevents the apoptosis of hypertrophic chondrocytes, decreases vascular invasion of the growth plate by endothelial cells, and diminishes longitudinal bone growth [2]. Similarly, VEGF levels are increased within the hypoxic fracture site, and recombinant VEGF increases the rate of fracture repair [44]. Further, genetic stabilization of HIF- α isoforms *via* deletion of *pVhl* promotes long bone growth partially through a VEGF-dependent mechanism [45]. At the cellular level, VEGF exerts pleiomorphic effects upon the cells present within bone. It acts as an anti-apoptotic factor to primary human osteoblasts [46], promotes the migration of osteoprogenitors and osteoblasts [47, 48], and increases in nodule formation and matrix mineralization [49-51].

VEGF signals *via* receptor tyrosine kinases VEGFR1 (Flt1), VEGFR2 (KDR in humans, Flk1 in mice), or VEGFR3 (Flt4); heterodimerization with co-receptors Neuropilin-1 or -2, or heparan sulfate proteoglycans, contributes to diversification of the biological response to ligand binding. We found that MC3T3-E1 cells express transcript for *VegfR1*, *Nrp1*, and, to a lesser degree, *Nrp2*. We observed no change in expression of any VEGF receptors during the course of 28-day osteogenic differentiation of MC3T3-E1 cells (data not shown). Deckers *et al.* have reported that the murine calvarial osteoblastic cell line KS483 increases expression of *VegfR1*, *VegfR2*, and *Nrp* in a differentiation-dependent manner [49]. In contrast, Harper *et al.* demonstrated that *Nrp-1* expression decreases during the course of osteogenic differentiation [52].

The stimulatory effect of VEGF upon endothelial cells has been shown to be mediated primarily through binding of VEGFR2 [53] specifically with reference to *AnxA2* [23]. In contrast, the effects of VEGF upon osteoblastic cells appear to be mediated primarily through VEGFR1. The selective VEGFR1 agonist PlGF mimicked the effect of VEGF₁₆₄ upon osteoblast migration, whereas the selective VEGF-R2 agonist VEGF-E was without effect upon osteoblast motility [47]. Similarly, PlGF and VEGF₁₆₄ both increase markers of osteoblast differentiation and matrix mineralization [46]. The anti-apoptotic effects of VEGF do not appear to be mediated through VEGF-R1 [46]. In our hands, MC3T3-E1 osteoblastic cells expressed transcript for *VegfR1*, *Nrp-1*, and, to a lesser extent, *Nrp-2*. Since agonism of

VEGF-R1 alone did not result in an increase in Annexin A2, we concluded that the influence of VEGF₁₆₄ upon *AnxA2* expression is mediated *via* heterodimerization of VEGFR1 and Neuropilin-1 or Neuropilin-2.

Physiologic oxygen tension varies *in vivo*: the pulmonary alveoli are exposed to near-atmospheric oxygen tension [54], whereas the deep zones of articular cartilage are nearly anoxic environs [55]. Measurements of intraosseous pressure within the femoral condyle [40], the femoral and humeral medullary cavities [37, 38], and subchondral bone [39] each indicate oxygen tensions of approximately 5%. Because bones are composed of various anatomic compartments, such as the trabeculae, Haversian systems, the endosteum and periosteum, and the marrow cavity, one may expect oxygen tensions to vary across these structural features. Indeed, Zahm *et al.* recently modeled oxygen distributions in cortical and cancellous bone and predicted an oxygen gradient across and along mature osteons and trabeculae [56]. Provided an oxygen tension of 95 mmHg (12.5%) at the arteriole end and 40mmHg (5.2%) at the venous end of the capillary, their model predicts that osteocytes in the second osteonal layer and beyond would be hypoxic. Thus, from this model and the measurements described above, it is apparent that there is a range of oxygen tensions within a healthy bone which produces both tissue-level normoxic and hypoxic zones. Further, the oxygen conditions within bone can also vary in response to, or cause, pathologic states. For example altered oxygen tension has been implicated in conditions of development[57], disuse [58] and skeletal fracture [59, 60]. Indeed the oxygen tension at a fracture site has been measured to be as low as 0.8% [41, 42]. We demonstrate in Figure 6 that osteoblastic cells are sensitive to slight changes in oxygen tension. Consistent with our previous works [61, 62], we demonstrate modest differences in cellular response (HIF-1 α stabilization or *AnxA2* transcript) between conditions of 21% oxygen (tissue culture normoxia) versus 5% oxygen (*in vivo* normoxia). Instead, the most striking changes occurred between 5% to 1% oxygen tension.

Within the musculoskeletal system, the pericellular environment is considered hypoxic during development and fracture repair. Provided the results described within, it is possible that VEGF-stimulated upregulation of *AnxA2* may be useful as Annexin A2 appears to play an important role in matrix mineralization [11, 12, 16]. Further, the bone marrow niche is hypoxic, and Annexin A2 expression by osteoblastic and endothelial cells is involved in the retention of hematopoietic stem cells (HSC) in the bone marrow niche by mediating osteoblast-HSC adhesion [63]. Further studies will be required to elucidate the requirement of VEGF for Annexin A2 expression in this variety of *in vivo* situations.

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A **B**

C

Figure 1. Hypoxia increases VEGF and Annexin A2 expression

(A) qPCR analysis of *Vegf* and *AnxA2* expression after 1, 3, 6, or 24h culture at 21% or 1% O₂. Data are first normalized to α-tubulin, then to expression at 21% O₂. (B) Western immunoblotting for Annexin A2 and α-tubulin after 24 hour culture at 21% or 1% O₂. (C) Quantitation of Annexin A2 protein expression after 24h culture at 21% or 1% O₂. Data are first normalized to α-tubulin then to expression at 21% O₂. Bars represent mean ± SEM, with a minimum of 3 technical replicates. ** indicates $p < 0.01$ compared to 21% O₂ control; *** indicates $p < 0.001$ compared to 21% O₂ control.

Figure 2. HIF- α stabilization promotes Annexin A2 expression

(A) Western immunoblotting for Annexin A2 and α -tubulin after 24 hour culture at 21% O_2 in the presence of 0, 50 and 100 μ M DFO. (B) Quantitation of Annexin A2 after 24h culture at 21% O_2 in the presence of 0, 50 and 100 μ M DFO. Data are first normalized to α -tubulin then to expression at 21% O_2 . Bars represent mean $\pm$ SEM, with a minimum of 3 technical replicates). * indicates $p < 0.05$ compared to 21% O_2 control.

A **B**

C **D**

E

Figure 3. VEGF₁₆₄ induces Annexin A2 expression

(A) VEGF₁₆₄ or VEGF₁₂₀ ELISA for conditioned media from MC3T3-E1 osteoblasts cultured for 6, 24, or 48 hours reveals significant increases in VEGF release after 24 or 48 hours of hypoxic culture compared to normoxic controls. (B) Addition of a VEGF-inhibiting antibody to cells cultured under hypoxia for 24 hours reveals attenuated induction of *AnxA2* mRNA. (C) Addition of VEGF₁₆₄ to cells cultured under normoxia for 6 hours reveals increases in *AnxA2* mRNA. (D) VEGF₁₆₄ increases Annexin A2 protein expression after 24 hour culture at 21% O₂. (E) Quantitation of Annexin A2 expression in response to VEGF₁₆₄. Bars represent mean $\pm$ SEM, from 3 technical replicates. * indicates $p < 0.05$ compared to vehicle; *** indicates $p < 0.001$ compared to vehicle.

A **B**

C

Figure 4. VEGF receptor isoform involvement in VEGF₁₆₄-induced Annexin A2 expression
(A) Expression of VEGF receptor isoforms in MC3T3-E1 osteoblastic cells and femora from 6-week old male mice. Data are normalized to α -tubulin. (B) Inhibition of VEGFR1 with 10 μ M CPO-B11 attenuated hypoxic induction of Annexin A2 protein expression. Data are first normalized to α -tubulin, then to expression at 21% O₂. (C) VEGF₁₂₀ does not mimic the ability of VEGF₁₆₄ to increase *AnxA2* transcription. Data are first normalized to α -tubulin, then to vehicle control. Bars represent mean $\pm$ SEM, from 3 technical replicates. * indicates $p < 0.05$ compared to vehicle.

A **B**

C

D

Figure 5. VEGF₁₆₄-induced Annexin A2 expression involves ERK1/2 and Src
 (A) Western immunoblotting shows that VEGF₁₆₄ (25 ng/mL) transiently increases Tyr204 phosphorylation of ERK1/2. (B) Quantitation of VEGF₁₆₄-induced changes in ERK1/2 phosphorylation. Data are first normalized to pERK and then to control at 0mins. (C) Src inhibition with PP2 prevents VEGF₁₆₄-induced ERK1/2 protein phosphorylation. Data are first normalized to pERK and then to control at 0mins. (D) Inhibition of Src or MEK prevents VEGF₁₆₄-induced AnxA2 transcription. Data are first normalized to α -tubulin, then

to vehicle control. Bars represent mean $\pm$ SEM, from 3 technical replicates. * indicates $p < 0.05$ compared to vehicle; ** indicates $p < 0.001$ compared to vehicle.

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A **B**

Figure 6. Expression of HIF-1α and *Anxa2* transcript at varying oxygen tensions
 (A) Western immunoblotting for HIF-1α after culture for 3 hours at 21%, 10%, 5%, or 1% O₂. (B) AnxA2 levels after 24 hour culture in 21%, 10%, 5%, or 1% O₂. Data are first normalized to α-tubulin, then to vehicle control. Bars represent mean±SEM, from 3 technical replicates. * indicates $p < 0.05$ compared to 21% oxygen.