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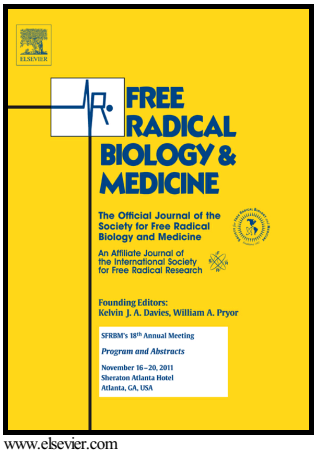

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Expression of human inducible nitric oxide synthase in response to cytokines is regulated by hypoxia-inducible factor-1

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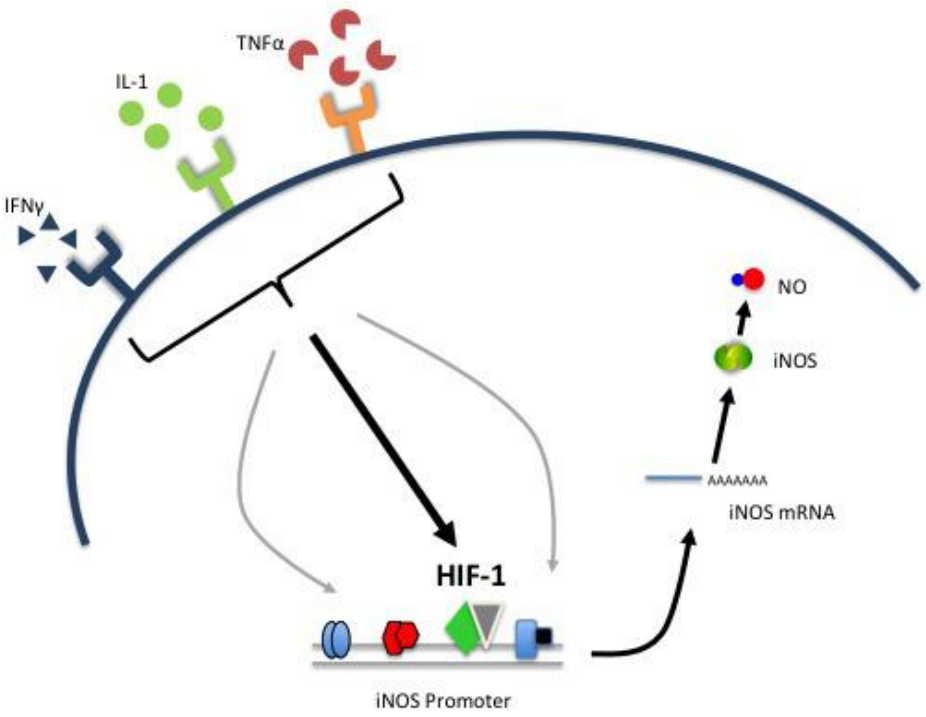
ABSTRACT

The production of nitric oxide (NO) by inducible NO synthase (iNOS) and the regulation of gene expression by hypoxia-inducible factors (HIFs) are important for many aspects of human cell biology. However, little is known about whether iNOS expression is controlled by HIFs in human cells. Stimulation of A549 human lung epithelial cells with cytokines (TNF, IL-1 and IFNγ) increased the nuclear accumulation of HIF-1 in normoxic conditions. Activation of HIF-1 by hypoxia or $CoCl_2$ was not sufficient to induce iNOS expression. However, pharmacological inhibition of HIF-1 reduced the induction of iNOS expression in A549 cells and primary human astrocytes. Moreover, elimination of HIF-1α expression and activity by CRISPR/Cas9 gene editing significantly reduced the induction of human iNOS gene promoter, mRNA and protein expression by cytokine stimulation. Three putative hypoxic response elements (HRE) are present within the human iNOS gene promoter and elimination of an HRE at -4981 bp reduced the induction of human iNOS promoter activity in response to cytokine stimulation. These findings establish an important role for HIF-1α in the induction of human iNOS gene expression in response to cytokine stimulation.

Graphical abstract



Abbreviations: **$CoCl_2$**, Cobalt chloride; **HIF**, Hypoxia-inducible factor; **HK-1**, Hexokinase-1; **HRE**, Hypoxia response element; **iNOS**, Inducible nitric oxide synthase; **NO**, Nitric oxide; **PHD**, prolyl hydroxylase; **sgRNA**, single-guide RNA; **VEGF-A**, Vascular endothelial growth factor-A

Keywords: Nitric oxide synthase, Nitric oxide, Gene regulation, Hypoxia-inducible factor (HIF), Cytokine

INTRODUCTION

Nitric oxide (NO) has broad ranging biological effects that control essential physiological processes and dysregulation of its production is involved in the development of many pathologies [1]. This bioactive gas is synthesized by a family of nitric oxide synthases (NOSs): neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). In contrast to the largely constitutive nature of nNOS and eNOS expression, iNOS expression and activity is tightly regulated to maintain precise NO levels that have effects on immune activation, inflammation and tissue damage [2]. iNOS is normally absent in unstimulated cells and expression is highly induced in response to inflammatory stimuli, such as

cytokines and microbial components [3]. Transcriptional induction of the human iNOS promoter requires the synergistic action of several transcription factors [4]. Stimulation of cells by cytokines that activate NF-κB and MAPK pathways results in the activation of NF-κB, AP-1 and C/EBPβ transcription factors that cooperatively drive iNOS gene expression [5-8]. Activation of STAT1 by interferon-γ (IFNγ) is also needed for maximal induction of this enzyme in human cells [9].

Hypoxia-inducible factors (HIFs) are central regulators of cellular responses to hypoxia and cellular metabolism [10]. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β/ARNT subunits [11]. Both subunits are actively expressed in normoxic conditions but HIF-1α is rapidly degraded because of prolyl hydroxylation by prolyl hydroxylases (PHDs) that leads to its ubiqutinylation by von Hippel Lindau protein and subsequent proteasome-mediated degradation [12]. In hypoxia, PHD activity is inhibited thereby preventing HIF-1α degradation and increasing its levels [13]. HIF-1 nuclear translocation and binding to HIF responsive elements (HREs) within gene promoters drives transcriptional activation of genes involved in tissue perfusion, cell survival and glycolytic metabolism that are needed for tissue protection in hypoxic conditions and regulation of inflammation [14, 15]. The induction of glycolytic genes by HIF-1 also controls the metabolic reprogramming of cells to increase glycolysis in normoxic conditions, a process termed aerobic glycolysis. Induction of aerobic glycolysis affects the regulation of immune responses and the pathogenesis of cancer [16, 17].

iNOS has been suggested to be a HIF-regulated gene. Specifically, the induction of iNOS by cytokines in murine macrophages is increased by hypoxia through an HRE-dependent mechanism [18, 19]. Cellular stresses, such as iron deprivation, also induce the HIF-dependent expression of iNOS in cooperation with IFNγ stimulation in murine cells [20]. However, there are important differences in the mechanisms by which iNOS expression is induced in human and murine cells. Specifically, the human iNOS gene promoter is structurally different than the murine counterpart, is regulated by distinct regulatory regions, and is methylated [21-23]. These differences may underlie the disparate

expression of iNOS in mouse and human cells. Cultured human macrophages do not induce iNOS expression in response to inflammatory cytokines and the cell type-specific activity of the human iNOS promoter is different than mouse iNOS expression within tissues *in vivo* [24, 25]. iNOS expression can be induced by cytokine stimulation in certain human cell lines, such A549 transformed lung epithelial cells, and primary cell types, such as astrocytes and bronchial lung epithelial cells [26, 27]. The synergistic action of multiple cytokines is needed for maximal induction of iNOS expression in most cell types.

We examined the role of HIF-1 in regulating the induction of human iNOS expression in normoxic conditions. The nuclear accumulation of HIF-1α was increased after stimulation of human cells with cytokines. Pharmacological inhibition of HIF-1 and CRISPR/Cas9-mediated elimination of HIF-1α reduced cytokine-mediated induction of human iNOS gene promoter activity, mRNA and protein expression. Further, there are 3 predicted HREs in the human iNOS gene promoter and mutational elimination of a site at -4981 bp reduced the induction of gene promoter activity by cytokines. Our findings identify HIF-1 as a cytokine-activated transcription factor that increases human iNOS expression.

MATERIAL AND METHODS

Cell culture

A549 human lung epithelial cells were purchased from ATCC and cultured in F-12K medium containing 10% FBS at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. Primary human astrocytes were purchased from Lonza, cultured in ABM medium (Lonza, Basel, Switzerland) supplemented with the AGM SingleQuot Kit (Lonza), and used between passages 4 and 12 for experiments. For all experiments using A549 cells, confluent cells were incubated in basal medium in the absence of serum for 24 h prior to cytokine treatment. To induce iNOS expression, cells were treated with a cytokine mixture of human recombinant IL-1α (10 ng/ml; PeproTech, Rocky Hill, NJ),

TNF (10 ng/ml; PeproTech), and IFNγ (100 ng/ml; Invitrogen) for the indicated times. This combination of cytokines maximally induces iNOS expression. Stimulation with individual cytokines has little effect. HIF-1α expression was inhibited by incubating cells with PX-478 (20 μM; Selleck Chemicals LLC, Houston, TX) for 16 h prior to cytokine stimulation. In some experiments cells were incubated in hypoxic conditions (1% O_2) for 24 h in a humidified CO_2 chamber. Chemical hypoxia was induced by treatment with 200 μM $CoCl_2$ for 5 h. All experiments were performed at least three times unless otherwise indicated.

SDS-PAGE and immunoblotting

Cells were lysed and cell lysates immunoblotted as described previously [28]. Primary antibodies used were: mouse monoclonal anti-HIF-1α (NB100-105; Novus Biologicals, Littleton, CO), rabbit monoclonal anti-HIF-1α (14179S; Cell Signaling Technology, Danvers, MA), rabbit monoclonal anti-iNOS (NBP1-33780; Novus Biologicals), mouse monoclonal anti-caspase-8 (MAB4708; EMD Millipore, Temecula, CA), rabbit polyclonal anti-Histone H2A (sc-67218; Santa Cruz, Dallas, TX), and mouse monoclonal anti-β-actin (A1978, Sigma-Aldrich, St. Louis, MO). Immunoblot images were quantified by densitometry using the National Institutes of Health ImageJ software, and protein expression data were normalized to β-actin levels or to appropriate loading controls for the quantification of proteins in subcellular fractions.

RNA Isolation and RT-qPCR

Total RNA was isolated from cells using a RNeasy mini kit as per the manufacturer's instructions (Qiagen) and TaqMan quantitative RT-PCR (RT-qPCR) was performed as described [29] using validated primer/probe sets for HIF-1α, VEGF-A, HK1, iNOS, GAPDH and MEK1 (Applied Biosystems, Foster City, CA). The relative expression of target genes was calculated by normalization

to GAPDH. In experiments involving alterations in HIF-1 expression, target gene expression was additionally normalized to MEK1 because some studies have reported that the expression of GAPDH may be regulated by HIF-1 in response to hypoxia [30]. We did not observe any change in GAPDH levels in our experiments but included additional normalization to MEK1 as a secondary analysis. Data were acquired on an ABI 7900HT fast real-time PCR system (Applied Biosystems).

Quantification of NO production

NO production was measured by quantifying the accumulation of nitrite in culture supernatants using a NO-specific chemiluminescence analyzer as described previously (Sievers, Boulder, CO) [28].

Transfection of mammalian cells

For iNOS promoter luciferase experiments, A549 cells were transfected with 1 μg of a human iNOS promoter-luciferase construct containing 7.2 kb of the human iNOS promoter upstream of the transcription start site (pXP2-iNOS; kindly provided by Dr. David Geller, University of Pittsburg, Pittsburg, PA[5]), human iNOS promoter-luciferase plasmids containing mutated HREs (pXP2-iNOSΔHRE), or pXP2 backbone plasmids using Lipofectamine 3000 reagent as per the manufacturer's instructions (Invitrogen, Carlsbad, CA). To control for transfection efficiency, cells were also transfected with a pRL-SV40 Renilla luciferase plasmid and firefly luciferase activity normalized to the levels of Renilla luciferase. Following transfection, cells were plated into 96-well flat bottom white plates, rested for 24 h in basal F-12K media, and then used for experiments.

For CRISPR-Cas9 experiments, A549 cells were harvested by trypsinization, and 1×10^6 cells were transfected with 1 μg of pSpCas9(HIF-1α)-2A-GFP plasmid encoding the HIF-1α guide RNA or 1 μg of the pSpCas9(BB)-2A-GFP backbone vector using Lipofectamine 3000 reagent as per the manufacturer's instructions (Invitrogen).

Luciferase reporter assay for iNOS promoter activity

A549 cells were transfected with iNOS promoter luciferase plasmids and a constitutively expressed Renilla luciferase construct (pRL-SV40). Cells were then stimulated with cytokines for 8 h and luciferase activity was measured using a Dual-Glo Luciferase Assay System as per the manufacturer's instructions (Promega, Madison, WI) and as described previously [31]. Luminescence was quantified using a Tecan Infinite 200 Pro multimode reader.

The sequence of the human iNOS promoter was queried for HRE consensus sequences (5'-(A/G)CGTG-3') using the motif-based sequence analysis tool FIMO (Find individual motif occurrences). Three putative HREs were identified and each was excised from the human iNOS promoter-luciferase plasmid using overlap extension PCR [32, 33]. Briefly, PCR was performed on the wild type human iNOS promoter-luciferase construct (pXP2-iNOS) using 2 primers flanking the 7.2 kb human iNOS promoter (pXP2_F 5'-GGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGGATCCCCAGGG-3', pXP2_R 5'-CGAACGTGTACATCGACTG-3') and pairs of overlapping primers at each putative HRE (HRE1_Mutagenesis primer 5'-GCAGAACAGCCAGGGGGCAGCCGCCCTCTCTG-3', HRE2_Mutagenesis primer 5'-GTGAAGGCCCCACAGGACACTCGCCACGGGACATGC-3', HRE3_mutagenesis primer 5'-CTTTCACTGAGGCTTCTCCTGTGTGCATCACAGACATTAAC-3'). The three mutated amplicons were then cloned into the parental pXP2-iNOS plasmid to generate three single HRE-deficient human iNOS promoter-luciferase reporter constructs (pXP2-iNOSΔHRE1, pXP2-iNOSΔHRE2, pXP2-iNOSΔHRE3). Successful elimination of HREs was confirmed by Sanger sequencing with primers flanking the three sites. (iNOS_HRE1_F 5'-TCTGGATCCCCAGGGCCCAGC-3', iNOS_HRE1_R 5'-GGCAACAGTGAGACTCTGATCTC-3'. iNOS_HRE2_F 5'-CACTTCCGTCATCTCC-3', iNOS_HRE2_R 5'-

CGGAGTTCCTACTGTGTGCC-3', iNOS_HRE3_F 5'-CCCACCTGATCCTCCTGAGT-3', iNOS_HRE3_R 5'-GGCCTCAAAGCACTTCGTTG-3'). A549 cells were transfected with the wild-type human iNOS promoter-luciferase construct or with the single HRE-deficient human iNOS promoter-luciferase constructs, and the luciferase reporter assay was carried out as described above.

CRISPR-Cas9 gene editing of HIF-1α

A 20 nucleotide, CRISPR RNA (crRNA) sequence (5'-TGGTATCATATACGTGAATG-3') targeting the human HIF-1α gene was designed using the online single-guide RNA (sgRNA) designer tool CRISPRko (Broad Institute). The HIF-1α_sgRNA (5'-CACCGTGGTATCATATACGTGAATG-3') (Integrated DNA Technologies) was cloned into pSpCas9(BB)-2A-GFP. A549 cells were transfected with pSpCas9(HIF-1α)-2A-GFP using Lipofectamine. Following transfection, GFP-positive cells were single cell sorted and clonal populations expanded in F-12K medium containing 10% FBS at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

To screen for Cas9-mediated gene editing of HIF-1α, a PCR reaction was carried out using the HIF-1α forward primer (5'-GGGTAAAGAACAAAACACACAGCGAAGC-3') and the HIF-1α_sgRNA reverse primer (5'-CACCGTGGTATCATATACGTGAATG-3'), the latter being complimentary to the unedited HIF-1α sequence. In this scenario, the reverse primer binds to the unedited HIF-1α sequence but not an edited version because it is not complimentary to the edited gene sequence. In addition to examining HIF-1α gene editing by PCR for the edited region, a region of the HIF1α gene was amplified by PCR using the HIF-1α forward primer (5'-GTGTACCCTAACTAGCCGAGGA-3') and the HIF-1α reverse primer (5'-TGGGGGCAGTAGTAGTACAGTTG-3') and the amplicon sent for Sanger sequencing (Genewiz). Further, HIF-1α protein expression was determined by treating cells with 200 μM $CoCl_2$ and immunoblotting for HIF-1α.

Statistical analyses

Unless otherwise indicated, quantitative data presented are the mean ± S.D. of at least three independent experiments. Statistical comparisons between two groups were made using a Student's test and between multiple groups with analysis of variance followed by a post-hoc multiple comparisons test. Statistically significant differences were defined as $p < 0.05$.

RESULTS

Cytokines induce nuclear translocation of HIF-1α

Protein levels of HIF-1α are tightly regulated in normoxic conditions by post-translational modification and proteasomal degradation. However, functional levels of this transcription factor can be present in normoxic conditions [34]. We examined the expression and cellular localization of HIF-1 in unstimulated and cytokine (TNF, IL-1, IFNγ) stimulated cells. A549 transformed human lung epithelial cells were unstimulated or stimulated with cytokines and the expression of HIF-1α was examined by immunoblotting. Basal HIF-1α levels were detectable in whole cell lysates and cytokine stimulation marginally affected the total levels of this protein up to 8 h post-stimulation (Fig. 1A). The transcriptional activity of HIF-1 depends on its translocation from the cytosol into the nucleus. As such, the subcellular localization of HIF-1α was assessed by fractionating cell lysates into cytosolic and nuclear components. Purity of the nuclear fraction was confirmed by high levels of histone H2A and undetectable amounts of caspase-8, and of the cytosolic fraction by high levels of caspase-8 and little histone H2A (Fig. 1B). The majority of HIF-1α was present in the cytosol although there was a low level of this HIF-1 subunit in the nucleus of unstimulated cells. Cytokine stimulation increased HIF-1α nuclear localization by 2 h post-stimulation and this persisted up to 8 h post-stimulation (Fig. 1B). This increase in HIF-1α nuclear localization was coincident with the kinetics of iNOS induction

after cytokine stimulation, in which iNOS protein expression was apparent at 3 - 4 h post-stimulation (Fig. 1C).

HIF-1 stabilization alone does not induce iNOS expression

We first examined whether HIF-1 activation is sufficient to induce iNOS expression. A549 cells were untreated or subjected to hypoxia for 24 h, cells lysed, and the expression of HIF-1α and iNOS determined by immunoblotting. Hypoxia increased HIF-1α levels but did not induce iNOS expression (Fig. 2A). Also, the induction of iNOS expression by cytokines was not potentiated by hypoxia (Supplemental Figure 1). Similarly, treatment of cells with the hypoxic mimetic $CoCl_2$ increased HIF-1α levels but did not induce iNOS expression (Fig. 2B). Thus, activation of HIF-1 alone is not sufficient to induce iNOS expression in human cells.

Pharmacological inhibition of HIF-1α reduces the induction of iNOS expression by cytokines

Because we observed that cytokine stimulation of cells resulted in the increased nuclear localization of HIF-1α, we examined whether this transcription factor is involved in the induction of iNOS expression in response to cytokine stimulation even though it was not sufficient for iNOS induction in response to hypoxia or $CoCl_2$. A549 cells were treated with PX-478, a pharmacological inhibitor of HIF-1α that prevents its expression [35, 36]. Consistent with its known effects, treatment of A549 cells with PX-478 markedly reduced HIF-1α mRNA and protein expression (Fig. 3A & B). The effect of PX-478 on iNOS gene promoter activity was assessed by transfecting cells with a human iNOS promoter-luciferase plasmid, treating cells with PX-478 for 16h, and then leaving cells unstimulated or stimulating them with cytokines. The expression of luciferase was quantified 8 h later. Cytokines induced human iNOS gene promoter activity and this was significantly reduced by PX-478 (Fig. 3C). Cells were then pre-treated with PX-478 and the effect on cytokine stimulation of iNOS

mRNA and protein expression was quantified. PX-478 significantly reduced the induction of human iNOS mRNA and protein expression (Fig. 3D & E). In addition to A549 cells, the induction of iNOS expression by cytokines was also significantly reduced by PX-478 in primary human astrocytes (Fig. 4).

Elimination of HIF-1α reduces the induction of iNOS expression by cytokines

The role of HIF-1 in the induction of human iNOS expression was then examined by eliminating HIF-1α using CRISPR/Cas9-mediated gene editing. Cells were transfected with a plasmid encoding Cas9, a guide RNA targeting the human HIF-1α gene, and eGFP. Transfected cells were isolated by single cell sorting of GFP-positive cells and clones expanded (Fig. 5A). Mutation of HIF-1α in transfected clones was evaluated by PCR for the Cas9-targeted region of the HIF-1α gene using primers specific for the wild-type non-edited sequence. In this scenario, primers bind to a non-edited allele but not the HIF-1α gene if it has been altered by Cas9-targeting. Using this approach, two cell clones that contained edited HIF-1α (HIF-1α-KO) were identified and one was chosen for further experimentation (Fig. 5B; clone 2). Disruption of the HIF-1α gene was confirmed by sequencing (data not shown). HIF-1α-KO cells also lacked protein expression of HIF-1α (Fig. 5C). The induction of HIF-regulated genes involved in the hypoxic response of cells was also assessed to determine HIF-1 function. Parental cells induced the expression of VEGF-A and HK-1 in response to the hypoxic mimetic $CoCl_2$ but the induction of these genes was completely absent in HIF-1α-KO cells (Fig. 5D and Supplemental Figure 2). Finally, NF-κB is essential for the induction of iNOS expression in response to cytokines and its activation is sensitive to oxidative conditions in cells [37]. IκBα degradation in response to cytokine stimulation was not affected in HIF-1α-KO cells, indicating that activation of the NF-κB pathway is not affected by the loss of HIF-1α in our experiments (Supplemental Figure 3).

The induction of iNOS in HIF-1α-KO cells was then examined. Parental and HIF-1α-KO cells were transfected with a human iNOS promoter-luciferase plasmid and stimulated with cytokines. There was significantly less induction of human iNOS promoter activity in HIF-1α-KO cells when compared to parental controls (Fig. 5E). When the expression of iNOS was examined, the induction of both iNOS mRNA and protein was reduced in HIF-1α-KO cells (Fig. 5F & G and Supplemental Figure 4). In addition, cytokine-induced NO production was also reduced in HIF-1α-KO cells (Fig. 5H). These data indicate that HIF-1α is needed for full induction of human iNOS gene expression in response to cytokines.

Cytokine-mediated iNOS gene promoter activity is induced by an HRE at -4981 bp

HIF-1 mediates its transcriptional activity by binding to HREs in the gene promoters of target genes. The presence of HREs in the human iNOS promoter is poorly characterized [18]. Three putative HREs were identified at -7188, -4981 and -2220 bp upstream of the transcription start site using FIMO (Fig. 6A). To determine the role of each HRE in the induction of iNOS expression by cytokines, each putative binding element was eliminated in human iNOS promoter-luciferase plasmids using site-directed excision. A549 cells were transfected with wild type human iNOS promoter-luciferase or each mutated plasmid, left unstimulated or stimulated with cytokines, and luciferase activity quantified. Cytokine induction of the human iNOS gene promoter was significantly reduced only when the HRE at -4981 bp was eliminated (Fig. 6B). No significant effects were observed when the putative HREs at -7188 and -2220 were mutated.

DISCUSSION

Studying the mechanisms by which iNOS expression is regulated in human cells has implications for understanding human physiology and development of several diseases. We have shown that cytokine induction of iNOS expression in human cells involves the transcriptional activity of HIF-1. This depends on the presence of an HRE at -4981 kb of the iNOS promoter. Several studies have shown previously that iNOS expression is induced in response to hypoxia but the specific role of HIF-1 in regulating iNOS in human cells has not been determined [38-41]. To our knowledge, this is the first report of the direct regulation of human iNOS expression by HIF-1 and provides new insight into the regulation of NO production and the biological effects of HIF-1.

HIF-1 is a central regulator of cellular responses to hypoxia through the up-regulation of genes that increase tissue perfusion, alter metabolic programming, and increase cell survival. In addition to hypoxia, inflammatory cytokines also affect the actions of HIF-1. Both TNF and IL-1 have been shown to increase the DNA binding and transcriptional activity of HIF-1, and this is associated with increased levels of HIF-1α in some studies but not others [42, 43]. The mechanism by which these cytokines increase HIF-1α levels depends on the activation of NF-κB that stabilizes protein levels of this HIF-1 subunit [44, 45]. Other inflammatory stimuli that activate NF-κB, such as microbial products that bind toll-like receptors, also increase HIF-1α expression [46]. In our studies, stimulation of A549 cells with cytokines increases nuclear accumulation of HIF-1α but did not significantly increase HIF-1α protein levels. This could differ from previous findings due to differences in the cell types used and/or the kinetics of HIF-1α expression examined. With regard to the latter, we focused on the effect of cytokines on HIF-1α levels early (within 8 h) after stimulation in order to examine processes related to the induction of gene transcription.

HIF-1α expression can be inhibited using pharmacological and genetic approaches. PX-478 is a selective inhibitor of HIF-1α expression and activity that has been examined as a potential therapy for cancer [47]. We observed that inhibiting HIF-1α with PX-478 reduced the induction of both iNOS

mRNA and protein expression by cytokines in A549 cells and primary human astrocytes. In addition to this, our studies also eliminated HIF-1α by CRISPR/Cas9-mediated gene editing. Genetic elimination of HIF-1α in this manner significantly reduced the induction of iNOS promoter activity as well as mRNA and protein expression by cytokines, confirming a role for this transcription factor in the induction of iNOS gene expression. Although CRISPR/Cas9 targeting of HIF-1α completely eliminated the induction of hypoxia-induced genes, there may be a small level of residual expression of HIF-1α protein in HIF-1α-KO cells after $CoCl_2$ exposure. A549 cells are a triploid cell line [48]. As such, editing of the HIF-1α gene may have differentially inactivated HIF-1α in each encoding allele by separate mechanisms such as eliminating protein expression or inactivating HIF-1α function despite the persistence of limited protein expression.

Mouse models have provided information on the biology of iNOS but there are differences in the mechanisms by which human and mouse iNOS expression is induced [24, 49, 50]. The iNOS gene promoters in human and mice are different. For instance, the inducibility of the human iNOS gene promoter depends on transcription factor binding sequences dispersed throughout 8.3 kb upstream of the transcription start site while the murine iNOS gene promoter is dependent largely on a 1.6 kb region [5, 6, 51, 52]. With regard to hypoxia and HIFs, several studies have shown that hypoxia alone is sufficient to induce the expression of iNOS in murine cells [53, 54]. This effect of hypoxia is likely to be context-dependent because other studies show that hypoxia is not sufficient to induce iNOS expression [55]. There is an HRE in the murine iNOS gene promoter located between 209 - 227 bp upstream of the start site [18]. We have observed that stabilization of HIF-1α alone is not sufficient to induce iNOS in human lung epithelial cells and that the HRE responsible for the induction of iNOS in response to cytokines is located at -4981 bp of the human iNOS promoter. These findings contribute to understanding how iNOS expression is specifically regulated in human cells and how this might differ from murine counterparts.

Understanding the mechanisms by which iNOS gene expression is regulated has implications for protective immunity and several pathological conditions. Production of iNOS by macrophages may be needed for the elimination of intracellular bacterial infections [56]. Recent evidence also indicates that the antimicrobial properties of macrophages depend on HIF-1 [57]. Also, HIFs increase the induction of aerobic glycolysis in macrophages, which is associated with development of a M1 macrophage phenotype that is protective against infection [58]. M1 macrophages are characterized by iNOS expression and our findings provide a potential link between HIF-1 activation and iNOS expression [59]. In addition to protective immunity, the induction of iNOS expression through a HIF-1-dependent mechanism protects against ischemic injury of the heart in murine models [60, 61]. Dysregulated activity of HIF-1 and of NO production are also involved in the development of several cancers. Expression of HIF-1α in tumor cells exacerbates angiogenesis and tumor growth [62]. Increased expression of iNOS is associated with the pathogenesis of certain tumors, and dysregulated production of NO has been experimentally determined to contribute to the progression and maintenance of several types of cancer [63-66]. As such, iNOS may be a downstream target of HIF-1 that is important in tumorigenesis.

In summary, we have identified a new transcriptional regulatory mechanism of iNOS in human cells. HIF-1α is needed for the maximal induction of iNOS expression in response to cytokines through amplification of human iNOS gene transcription. The findings provide insight into the role of iNOS in human biology and expand on developing knowledge of the cell biological roles of HIF in normoxia.

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FIGURE LEGENDS

Figure 1. Cytokine stimulation increases HIF-1α nuclear localization. A. A549 cells were stimulated with cytokines (CM: TNF, IL-1, IFNγ), lysed at the indicated time-points, and HIF-1α expression examined by immunoblot. **B.** A549 cells were untreated (UNT) or treated with cytokines, lysed at 2 h or 8 h post-stimulation, and lysates fractionated into cytosolic and nuclear components. HIF-1α levels were examined by immunoblot. **C.** A549 cells were stimulated with cytokines, lysed at the indicated time-points, and iNOS protein expression examined by immunoblot. * $p < 0.05$. ** $p < 0.01$.

Figure 2. Stabilization of HIF-1α alone is not sufficient to induce human iNOS expression. A549 cells were **A.** subjected to hypoxia for 24 h, **B.** treated with the hypoxic mimetic $CoCl_2$ for 5 h, or

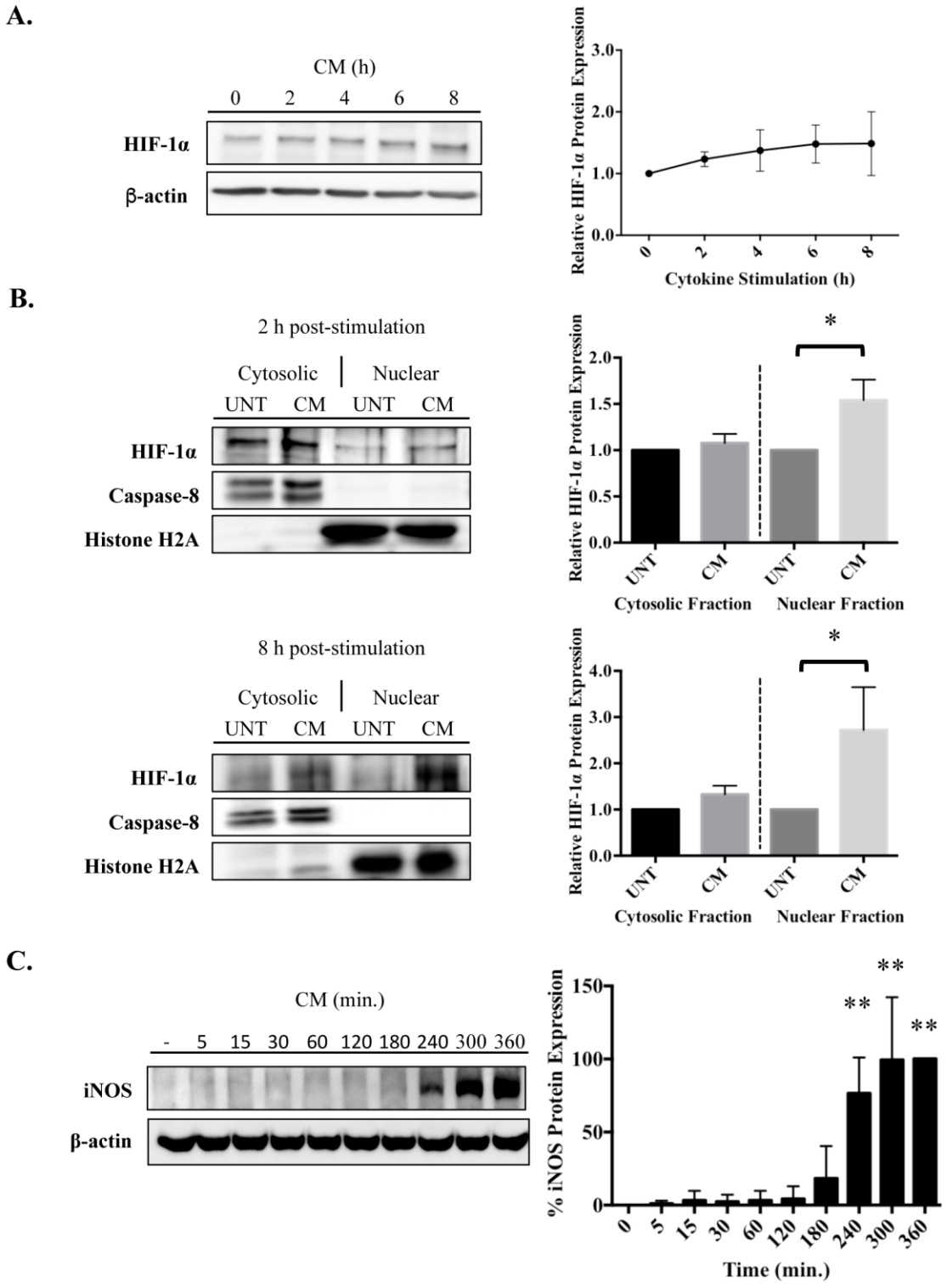
stimulated with cytokines (CM) for 5 h. Cells were lysed and the expression of HIF-1α and iNOS determined by immunoblot. Data shown is representative of 3 independent experiments.

Figure 3. Pharmacological inhibition of HIF-1α reduces cytokine-mediated induction of iNOS expression in A549 cells. A & B. A549 cells were treated with the HIF-1α inhibitor PX-478 overnight and left untreated (UNT) or stimulated with cytokines (CM). **A.** HIF-1α mRNA expression was quantified by RT-qPCR 2 h post-stimulation. **B**. HIF-1α protein expression was determined by immunoblot at 6 h post-stimulation. **C.** Cells were transfected with an empty control plasmid (pXP2) or a human iNOS promoter-luciferase plasmid (pXP2-iNOS). Cells were untreated or treated with PX-478 prior to stimulation with cytokines. The induction of luciferase activity was quantified 8 h post-stimulation. **D & E.** Cells were untreated or treated with PX-478 and stimulated with cytokines. **D.** iNOS mRNA expression was quantified by RT-qPCR 2h post-stimulation. **E.** iNOS protein expression was examined by immunoblot at 6h post-stimulation * $p < 0.05$, *** $p < 0.001$.

Figure 4. Pharmacological inhibition of HIF-1α reduces cytokine-mediated induction of iNOS expression in primary human astrocytes. Primary human astrocytes were untreated or treated with PX-478 prior to stimulation with cytokines (CM). Cells were lysed after 6 h and iNOS expression determined by immunoblot. ** $p < 0.01$

Figure 5. CRISPR/Cas9-mediated gene editing of HIF-1α reduces cytokine-mediated induction of iNOS expression. A. Schematic of CRISPR/Cas9-mediated gene editing of HIF-1α. **B.** DNA was isolated from cell clones that had been transfected with a plasmid not encoding a sgRNA or with a plasmid containing a HIF-1α sgRNA. PCR using primers complimentary to the sgRNA-targeted sequence in HIF-1α was performed to detect the presence of non-edited alleles. **C.** Parental A549 cells (WT) and a clone containing edited alleles of HIF-1α (HIF-1α-KO; clone 2) were stimulated with $CoCl_2$ and HIF-1α expression determined by Western blot. Western blot shown is representative of 2 independent experiments. **D.** Parental A549 cells and HIF-1α-KO cells were treated with $CoCl_2$ overnight and the mRNA expression of HIF-regulated genes VEGF-A and HK-1 quantified by RT-qPCR. Graph is the mean ± SD of triplicate measurements in one of two replicate experiments. **E.** Parental A549 cells and HIF-1α-KO cells were transfected with an empty control plasmid (pXP2) or a human iNOS promoter luciferase plasmid (pXP2-iNOS). Cells were stimulated with cytokines (CM) and luciferase activity quantified. **F-H.** Parental A549 cells and HIF-1α-KO cells were stimulated with cytokines. **F.** iNOS mRNA expression was quantified by RT-qPCR. **G.** iNOS protein expression determined by immunoblot. **H.** NO production was determined by quantification of nitrite levels in the supernatant. * $p < 0.05$, *** $p < 0.001$.

Figure 6. An HRE at -4981 bp of the human iNOS promoter induces iNOS promoter activity in response to cytokines. A. Schematic representation of the location of putative HREs in the human iNOS promoter and the human iNOS promoter luciferase plasmids that were generated to eliminate each of the HREs. **B.** A549 cells were transfected with plasmids encoding human iNOS promoter-luciferase or the iNOS promoters in which each of the putative HREs were eliminated. Cells were stimulated with cytokines (CM) and luciferase activity quantified. * $p < 0.05$.



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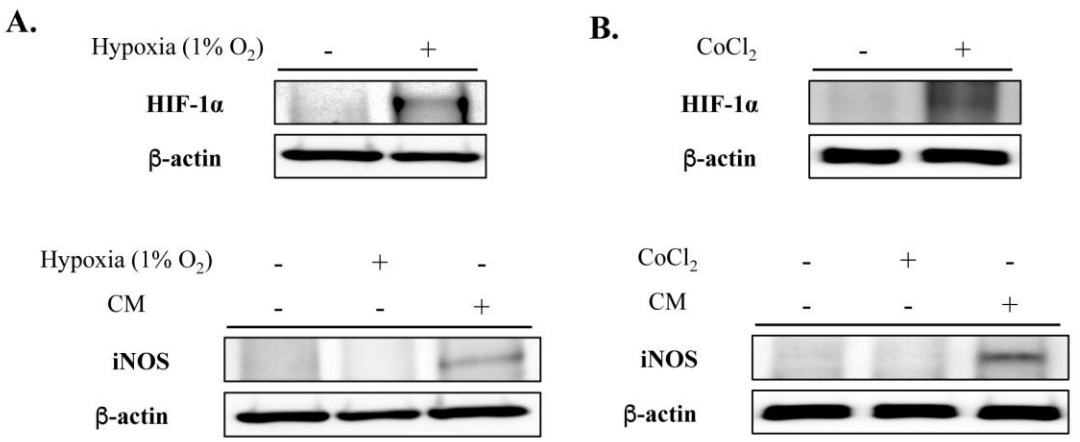

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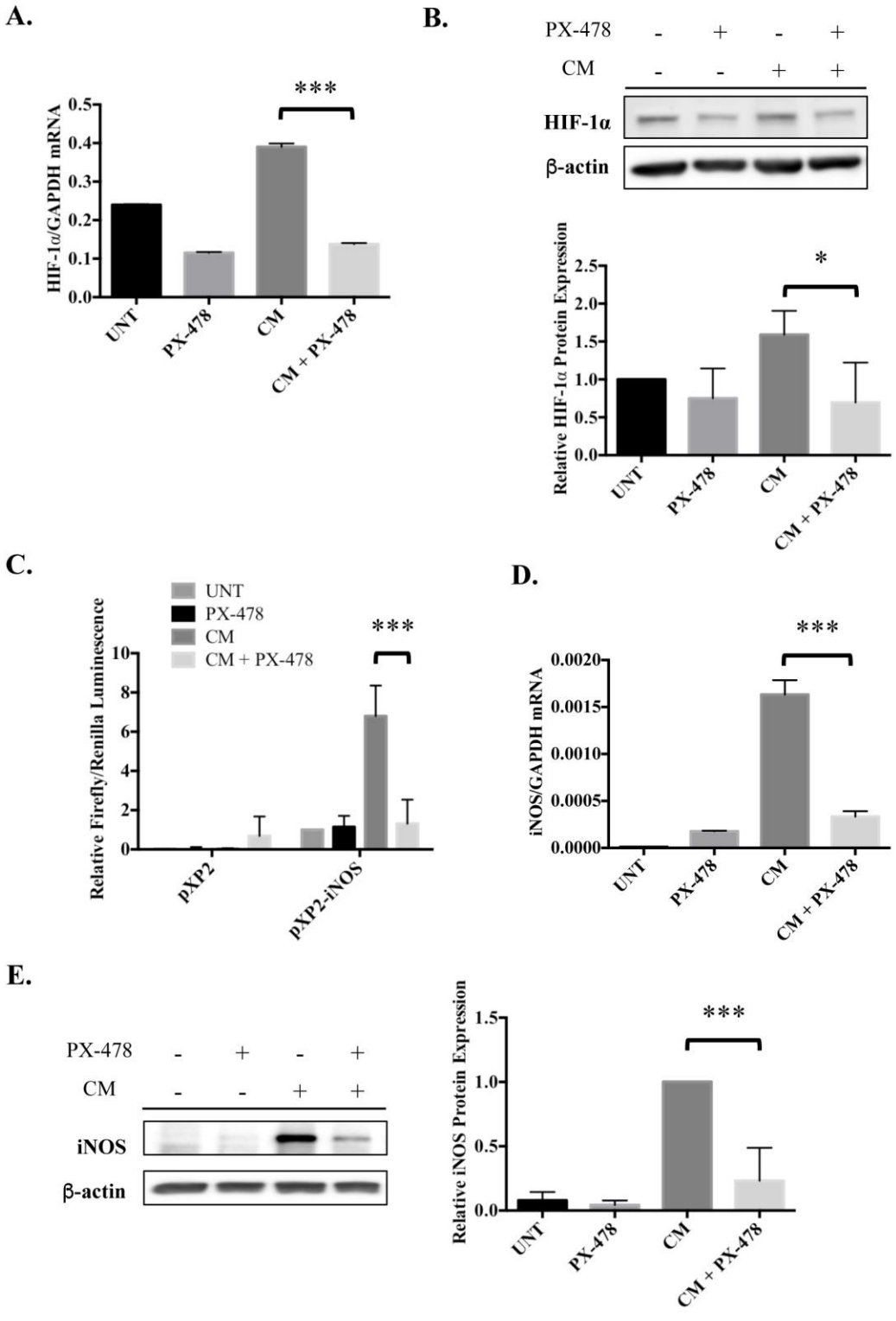
A.
HIF-1α/GAPDH mRNA
0.5
0.4
0.3
0.2
0.1
0.0
***
UNT
PX-478
CM
CM + PX-478
B.
PX-478 - + - +
CM - - + +
HIF-1α
β-actin
Relative HIF-1α Protein Expression
2.5
2.0
1.5
1.0
0.5
0.0
*
UNT
PX-478
CM
CM + PX-478
C.
UNT
PX-478
CM
CM + PX-478
Relative Firefly/Renilla Luminescence
10
8
6
4
2
0
***
pXP2
pXP2-iNOS
D.
iNOS/GAPDH mRNA
0.0020
0.0015
0.0010
0.0005
0.0000
***
UNT
PX-478
CM
CM + PX-478
E.
PX-478 - + - +
CM - - + +
iNOS
β-actin
Relative iNOS Protein Expression
1.5
1.0
0.5
0.0
***
UNT
PX-478
CM
CM + PX-478

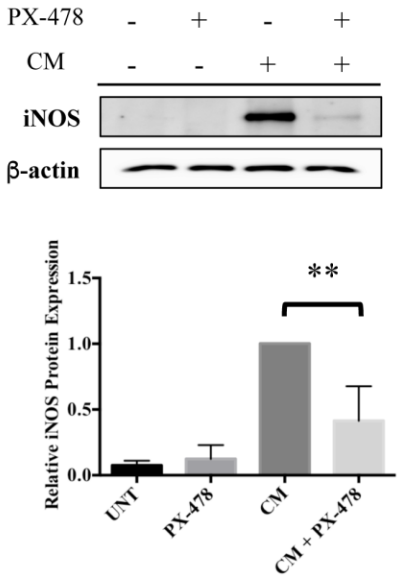

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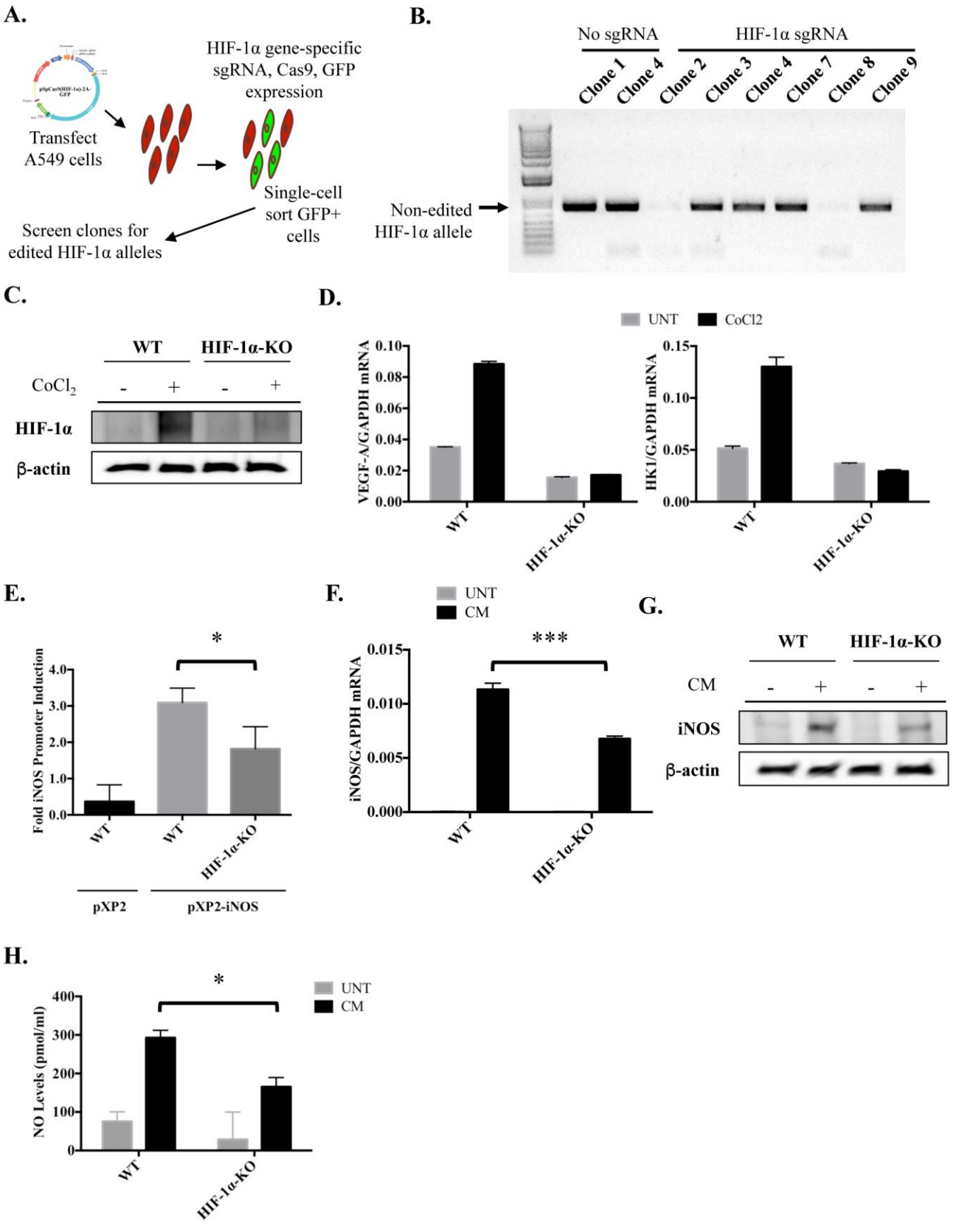
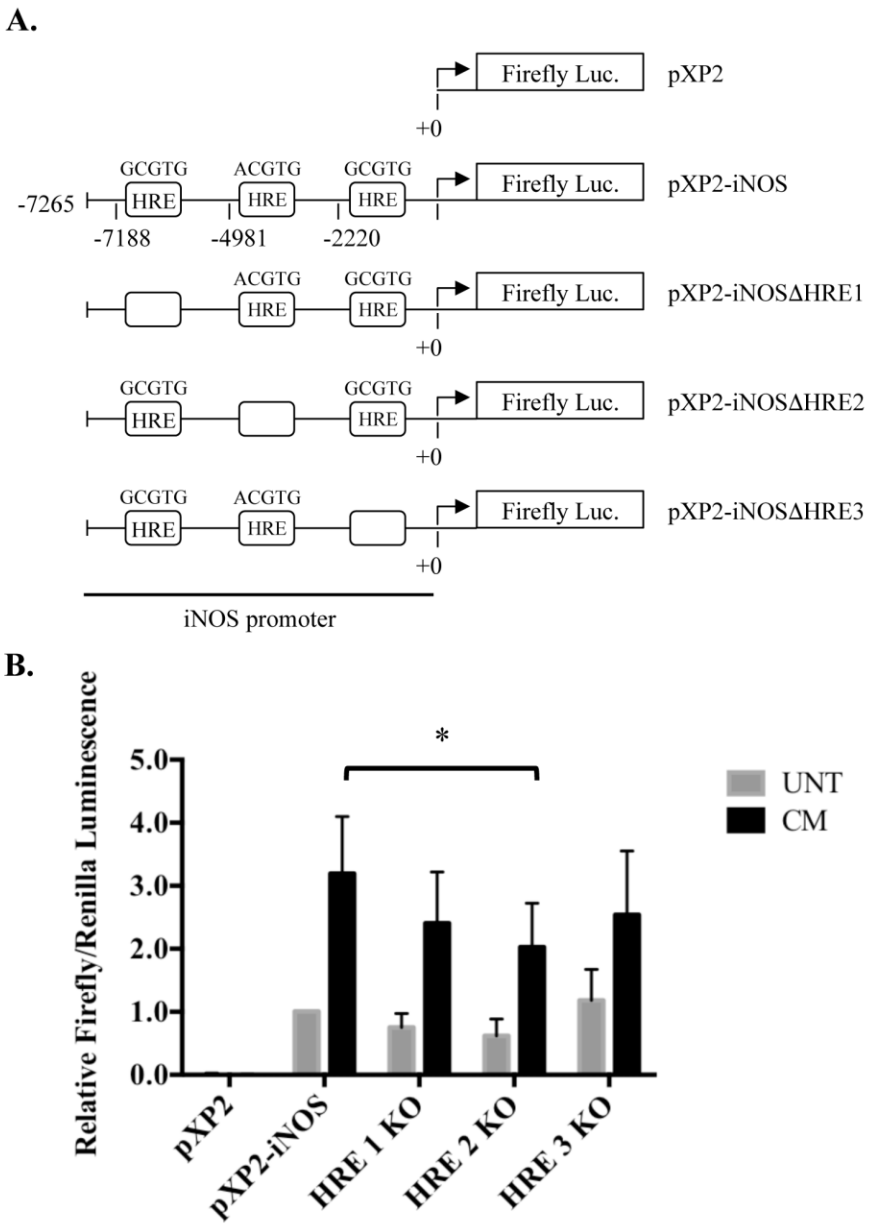
Figure 4 - Lee et al.


A.
HIF-1α gene-specific sgRNA, Cas9, GFP expression
Transfect A549 cells
Single-cell sort GFP+ cells
Screen clones for edited HIF-1α alleles
B.
No sgRNA
HIF-1α sgRNA
Clone 1
Clone 4
Clone 2
Clone 3
Clone 4
Clone 7
Clone 8
Clone 9
Non-edited HIF-1α allele
C.
WT
HIF-1α-KO
CoCl2
HIF-1α
β-actin
D.
UNT
CoCl2
VEGF-A/GAPDH mRNA
HK1/GAPDH mRNA
WT
HIF-1α-KO
E.
Fold iNOS Promoter Induction
pXP2
pXP2-iNOS
F.
UNT
CM
iNOS/GAPDH mRNA
G.
CM
iNOS
β-actin
H.
NO Levels (pmol/ml)



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Highlights

- Cytokines induce nuclear translocation of HIF-1α in normoxia
- HIF-1 is needed for maximal induction of human iNOS expression
- There is a functional hypoxia response element at -4981 bp of the human iNOS gene promoter