Published in final edited form as:

Cancer Res. 2008 April 15; 68(8): 2781–2788. doi:10.1158/0008-5472.CAN-07-2635.

Manganese Superoxide Dismutase Modulates Hypoxia Inducible Factor-1 alpha Induction *via* Superoxide

Suwimol Kaewpila, Sujatha Venkataraman, Garry R. Buettner, and Larry W. Oberley

The Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, The University of Iowa, Iowa City, IA, 52242-1181

Abstract

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that plays an important role in O₂ homeostasis. Numerous observations suggest that changes in reactive oxygen species affect HIF-1 α stabilization and HIF-1 α transcriptional activation in many cell types. The antioxidant enzyme manganese superoxide dismutase (MnSOD) modulates the cellular redox environment by converting superoxide (O₂^{•−}) to hydrogen peroxide and dioxygen. Previous results from our group have demonstrated that overexpression of MnSOD in MCF-7 cells alters stabilization of HIF-1 α under hypoxic conditions; however, the underlying mechanism(s) is not known. Here we tested the hypothesis that MnSOD regulates the expression of HIF-1 α by modulating the steady-state level of O₂^{•−}. We found that decreasing MnSOD with siRNA in MCF-7 cells resulted in: 1) an associated increase in the hypoxic accumulation of HIF-1 α immunoreactive protein; 2) a significant increase in the levels of O₂^{•−} ($p < 0.01$); but 3) no significant change in the steady-state level of H₂O₂. Removal of O₂^{•−} using spin traps (α -4-pyridyl-1-oxide-N-*tert*-butylnitron and 5,5-dimethyl-1-pyrroline N-oxide) or the O₂^{•−} scavenger Tempol or an SOD mimic (AEOL10113) resulted in a decrease in HIF-1 α protein, consistent with the hypothesis that O₂^{•−} is an important molecular effector responsible for hypoxic stabilization of HIF-1 α . The evidence from both genetic and pharmaceutical manipulation is consistent with our hypothesis that O₂^{•−} can contribute to the stabilization of HIF-1 α .

Keywords

Hypoxia; MnSOD; HIF-1 α ; Superoxide; and siRNA

INTRODUCTION

The transcription factor hypoxia-inducible factor (HIF) is a key regulator of the cellular response to O₂ homeostasis. HIF up-regulates the expression of many genes including those responsible for angiogenesis, glycolysis, cell growth, cell survival, and metastasis [1,2]. HIF is a heterodimer composed of a constitutively expressed β subunit, and an oxygen-regulated α subunit [3,4]. There are three known forms of HIF: HIF-1, HIF-2, and HIF-3. The immediate response to hypoxia is principally mediated through an increase in the level of HIF-1 α , a ubiquitously expressed protein in most cell types. When O₂ is adequate, two prolyl residues at the N-terminal activation domain of HIF-1 α are targeted for hydroxylation by appropriate prolyl hydroxylase domain-containing proteins (PHDs). Upon hydroxylation, HIF-1 α binds to the Von Hippel-Lindau (pVHL) tumor suppressor protein, leads to its ubiquitination and

subsequent degradation *via* the 26s proteasome [5–7]. When there is inadequate O₂ in the cell for this hydroxylation reaction, HIF-1 α does not bind to pVHL; thus, it accumulates and translocates to the nucleus where it dimerizes with HIF-1 β leading to formation of the transcription factor HIF. HIF will bind to hypoxia responsive elements (HREs) within genes initiating their expression, for example vascular endothelial growth factor (VEGF) and erythropoietin (EPO) [8].

MnSOD is a primary antioxidant enzyme (AE) that localizes in the mitochondrial matrix of eukaryotes. MnSOD is essential for maintaining normal tissue function. It modulates the intracellular redox environment by dismutating O₂^{•−} produced by the electron transfer chain in mitochondria forming H₂O₂ and O₂: O₂^{•−} + O₂^{•−} + 2H⁺ → H₂O₂ + O₂. The majority of tumors have greater steady-state levels of O₂^{•−} due to loss of MnSOD [9]. Multiple studies have demonstrated that reactive oxygen species (ROS) generated from mitochondria can participate in the hypoxia signal transduction pathway that mediates HIF-1 α stabilization [10–12]. Lower levels of MnSOD protein and its activity have been found in many types of tumors [13–14], and one of such sample is MCF-7 cells [15]. Moderate overexpression of MnSOD in MCF-7 cells has been shown to suppress hypoxic accumulation of HIF-1 α protein at both 1% and 4% O₂ [16]. The downstream effects of HIF-1 α suppression by elevated levels of MnSOD activity resulted in a decrease in the secretion of VEGF protein in cells exposed to 1% O₂ [16]. Alternatively, overexpressing MnSOD or CuZnSOD in A549 human lung epithelial cells does not alter HIF-1 α stabilization under hypoxic conditions, while overexpressing GPx1 or catalase decreased HIF-1 α accumulation at low O₂ levels [17]. In both of these latter studies, changes in the levels of ROS were not reported.

Although the effects of MnSOD on HIF-1 α stabilization have been reported, the mechanism underlying MnSOD-mediated HIF-1 α regulation and the impact of ROS-removal on HIF-1 α in response to hypoxia have not been clearly defined. We hypothesize that MnSOD affects the expression of redox-sensitive genes, including HIF-1 α , by modulating ROS levels in cells. We used molecular genetic and chemical approaches for ROS manipulation to analyze the regulation of HIF-1 α during hypoxia. We observed that decreasing the level of MnSOD by siRNA transfection elevated the levels of O₂^{•−} and induced the accumulation of HIF-1 α . This induction of HIF-1 α was suppressed when O₂^{•−} was removed using O₂^{•−} scavengers or a SOD mimic. Here we propose that MnSOD plays an important role in regulating the accumulation of HIF-1 α during hypoxia by modulating the level of O₂^{•−}.

Materials and Methods

Reagents and chemicals

AEOL10113 (manganese (III) *meso*-tetrakis (N-ethylpyridinium-2-yl) porphyrin; MnTE-2-Pyp⁵⁺) was a gift from Dr. James D. Crapo, National Jewish Medical Research Center, Denver, CO. 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) was from Dojindo, Japan. *para*-hydroxy phenyl acetic acid (pHPA), horseradish peroxidase (HRP), α -4-Pyridyl-1-oxide-N-*tert*-butylnitron (POBN) and 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (Tempol) were from Sigma (St. Louis, MO).

Cell culture

Human immortalized non-malignant mammary epithelial cells, MCF10A, were cultured in Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12 (1:1)) supplemented with 5% horse serum, 20 ng/mL epidermal growth factor, 0.01 mg/mL insulin, and 500 ng/mL hydrocortisone. Human breast adenocarcinoma MCF-7 cells were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1 mM

sodium pyruvate, and 0.1 mM nonessential amino acids. Cells were routinely maintained at 37°C in a humidified atmosphere with 5% CO₂.

Induction of hypoxia

Cells were seeded into 60 mm culture dishes; fresh medium was provided before hypoxic or chemical treatments. For hypoxia experiments the dishes were placed in a hypoxic chamber (Billups-Rothenberg, Del Mar, CA) and flushed with 1% O₂ (premixed 1% O₂, 5% CO₂, 94% N₂) for 5 min at 20 L/min, then the gas-exchange ports were closed and the chamber was placed in an incubator at 37°C for 4 h.

Inhibition of MnSOD by RNA interference

The pre-designed double stranded siRNA and its complement directed against MnSOD (5'-GGCCUGAUUAUCUAAAAGCTT-') and the nonspecific siRNA, the commercially available nontargeting siRNA and its complement were purchased from Ambion Inc. (Austin, TX). Briefly, 1×10^6 cells were seeded into 60 mm dishes the day before transfection. After 24 h, the media was replaced with OptiMEM (Gibco). Cells were then transfected with siRNA using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instructions. After 24 h, the transfection media was replaced with regular complete media without antibiotics. After 72 h, cells were harvested or treated with hypoxia for further experiments.

Protein harvest for HIF-1 α

Medium was removed from tissue culture dishes and cells were rinsed twice with cold PBS, then aspirated. Boiling lysis buffer (1% SDS, 1 mM sodium *ortho*-vanadate, and 10 mM Tris buffer pH 7.4) was added to the cells [16]. Cells were quickly scraped and transferred into micro-centrifuge tubes and boiled for 5 min. The viscosity was reduced by passing the lysates through a 25 gauge needle, then centrifuging at 12,000 g, 4°C for 10 min and the supernatants transferred to a new tube. Protein concentration was determined with Bio-Rad DC protein assay.

Western blot analysis

Analysis of HIF-1 α protein used 4 – 20% gradient Tris-HCl polyacrylamide ready-to-use gels (Bio-Rad) and electrotransferred onto a PVDF membrane. Mouse monoclonal antibody to HIF-1 α (Pharmingen/Transduction Laboratories, San Diego, CA) was used as a primary antibody while mouse monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ambion, Austin, TX) was used as a primary antibody for loading control protein. Goat anti-mouse IgG (Pharmingen/Transduction Laboratories, San Diego, CA) was used as a secondary antibody against both primary antibodies. SDS-polyacrylamide gel (12%) was used for MnSOD western blot analysis. Equal protein loading was confirmed on immunoblots using rabbit anti-actin antibody (Sigma, St Louis, MO). Bands were visualized by chemiluminescence (Pierce, Rockford, IL). All immunoblots were determined from at least three separate experiments. Quantification of band intensity for HIF-1 α was determined using Alpha Imager 2200 program based on integrated density value of each HIF band normalized to GAPDH.

Antioxidant enzyme activity gels

MnSOD activity was visualized by native polyacrylamide gel electrophoresis, which is based on the inhibition by SOD of the in-gel reduction of nitroblue tetrazolium (NBT) [18]. Briefly, cells were washed with phosphate-buffered saline (pH 7.4) and lysates were prepared in NP40 lysis buffer (150 mM NaCl, 1% Nonidet-P40, and 50 mM Tris buffer, pH 8.0). Proteins were quantified using the Bio-Rad protein assay. Equal amounts of protein from different samples

were loaded onto a polyacrylamide gel (12% running gel with a 5% stacking gel). After electrophoresis, gels were stained with 2.43 mM NBT for 20 min in the dark, then rinsed with distilled water and 28 μ M riboflavin/28 mM TEMED (N,N,N',N'-tetramethylethylenediamine) was added and illuminated under fluorescent light. For catalase and glutathione peroxidase GPx activity, 8 and 10% running gel was used. For catalase activity, gels were incubated with 0.003% H₂O₂ for 10 min and then stained with 2% ferric chloride 2% potassium ferricyanide solution [19]. For GPx activity, gels were soaked in 1 mM GSH for 30 min and incubated with 0.008% cumene hydroperoxide for 10 min and then finally stained with 1% ferric chloride 1% potassium ferricyanide solution [19].

Antioxidant enzyme activity assays

SOD activity was also measured by the modified NBT method as described previously [20]. Briefly, SOD activity was determined spectrophotometrically at 560 nm by measuring the reduction of NBT. The O₂^{•-} generated from the xanthine and xanthine oxidase system reduces NBT. The reduction of NBT is competitively inhibited in the presence of SOD. The amount of protein that inhibits the reduction of NBT to 50% of maximum is defined as one unit of SOD activity. MnSOD activity was determined in the presence of 5 mM sodium cyanide. CuZnSOD activity was calculated by subtracting MnSOD activity from total SOD activity.

Superoxide radical anion formation in cultured cells

Electron paramagnetic resonance (EPR) spin trapping with DMPO was used to detect O₂^{•-}. This technique involves an addition reaction of a short-lived radical to a diamagnetic compound (spin trap) to form a more stable free radical product (spin adduct), which can be studied by EPR. The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped; the hyperfine couplings of the spin adduct are characteristic of the original trapped radical. In brief, cells were washed with PBS and incubated with 100 mM DMPO in chelated-PBS [21] (pH 7.4) for 15 min. The cells were then transferred to a TM quartz flat cell and EPR spectra were recorded using a Bruker EMX spectrometer equipped with a TM cavity. EPR spectra were obtained as an average of 15 scans with a modulation amplitude of 1 G; scan rate 80 G/81 s; receiver gain 10⁴ – 10⁶; microwave power, 40 mW; and modulation frequency of 100 kHz. The EPR peak heights are in arbitrary units.

Hydrogen peroxide determination

Extracellular H₂O₂ released from MCF-7 cells was measured by a fluorometric assay using *p*HPA in the presence of HRP as previously described [22]. Briefly, this method utilized the fact that H₂O₂ reacts with HRP forming compound I, which in turn reacts with *p*HPA forming a stable fluorescent dimer, [*p*HPA]₂. Cell medium was removed and the cell monolayer was washed three times with HBSS buffer. The medium was then replaced with phenol red-free HBSS (1.0 mL) supplemented with 6.5 mM glucose, 1 mM HEPES, 6 mM sodium bicarbonate, 1.6 mM *p*HPA, and 95 μ g/mL HRP. The H₂O₂ was allowed to accumulate in the modified HBSS for 1 h. The released H₂O₂ was followed spectrofluorometrically by measuring the dimer formed at excitation and emission wavelengths of 323 and 400 nm, respectively. The fluorescence intensity of each sample was corrected for any changes in pH and compared with standard concentrations of H₂O₂ determined by absorbance at 240 nm.

Clonogenic survival

Cells were washed, trypsinized, and plated immediately after hypoxia treatment with and without chemical agent treatments into 60 mm dishes. The dishes were maintained in an incubator at 21% O₂ for 14 days to allow colony formation. The colonies were fixed with 70% ethanol for 5 min and stained with Coomassie blue for 5 min. Those colonies containing greater than 50 cells were scored. Cell survival fraction (SF) was calculated as follows: SF = colonies

formed/(cells seeded $\times$ PE), where PE is plating efficiency, *i.e.* (number of colonies formed)/(number of cells seeded) $\times$ 100.

Statistic analysis

Data are mean $\pm$ standard error of mean (SEM) from three independent experiments. Statistical analyses to determine the differences between means were performed using one-way ANOVA, followed by a post hoc Tukey test, or Student's *t*-test. $p < 0.05$ was considered as statistically significant.

RESULTS

MnSOD protein expression was suppressed by transient siRNA transfection

To determine whether MnSOD could affect the expression of HIF-1 α under hypoxic conditions, we first manipulated MnSOD levels in human breast adenocarcinoma MCF-7 cells using specific RNA interference. The protein level of MnSOD in MCF-7 cells was observed to be lower than those of immortalized, non-malignant MCF10A breast cells (Fig 1A). In MCF-7 cells MnSOD protein expression was found to be suppressed by siRNA in a time- and concentration-dependent manner (Fig 1B–C). siRNA against MnSOD showed suppression of protein within 24 h after transfection with maximal decrease at 72 h. Nontargeting siRNA transfected cells were similar to untransfected control (**Neg** in Fig 1B–C). The transfection conditions of 300 pmol for 72 h were selected for use in all subsequent experiments. The activity of MnSOD in cells transfected with siRNA was below the limit of detection of the spectroscopic-based assay. However, the suppression of the activity of MnSOD could be shown by nondissociating native gel electrophoresis (Fig 1D). No changes in the activities of other AEs, such as CuZnSOD, catalase, or GPx as measured by activity gels were observed (data not shown).

Inhibition of MnSOD by siRNA increased O₂^{•−} levels and induced HIF-1 α accumulation in cells exposed to 1% O₂

We next determined the level of HIF-1 α protein induction in MCF-7 cells after being transiently transfected with siRNA against MnSOD, nontargeting siRNA, or exposure to the transfection agent alone. The transfected cells were exposed to 1% O₂ for 4 h and the relative levels of MnSOD protein were determined. Following treatment with MnSOD siRNA relative to nontargeting siRNA or transfection reagent control, a decrease in MnSOD protein and a modest increase of HIF-1 α protein was observed (Fig 2A). This was further confirmed by the quantitation of the blot intensities. These results suggest that changes in MnSOD levels had an effect on HIF-1 α accumulation under hypoxic conditions in MCF-7 cells.

To evaluate the functional consequences of MnSOD-mediated O₂^{•−} induction of HIF-1 α , we determined whether inhibiting MnSOD actually results in an increased level of O₂^{•−}, the substrate of MnSOD. MCF-7 cells were transfected with siRNA followed by exposure to 1% O₂. Free radical production was assessed by EPR by measuring the intensities of the DMPO-OH spin adduct, which corresponds to the relative rates of O₂^{•−} formation, which is inhibitable by SOD (not shown). After 4 h of hypoxia, cells accumulated EPR-detectable DMPO-OH adducts (Fig 2B). EPR spectra from cells transfected with MnSOD siRNA showed a greater peak height of the DMPO-OH spin adduct relative to nontargeting siRNA and untransfected controls. There were no significant differences in the intensities of the spectra observed from nontargeting siRNA and untransfected control. Quantitation of the spectral peak heights normalized to the amount of protein indicated a significant increase in accumulation of DMPO-OH measured from cells transfected with MnSOD siRNA relative to siNeg, $p < 0.01$ (Fig 2C). Extracellular accumulation of H₂O₂ from MnSOD siRNA transfected cells showed no significant difference relative to siNeg transfected cells (Fig 2D). These results demonstrate

that the suppression of MnSOD by siRNA significantly increased the level of $O_2^{\bullet-}$ and concomitantly increased HIF-1 α induction under hypoxia in MCF-7 cells. The increase in accumulation of HIF-1 α protein when MnSOD is decreased in this cell line suggests that $O_2^{\bullet-}$ plays a role in the regulation of HIF-1 α .

Scavenging of $O_2^{\bullet-}$ by the spin traps POBN and DMPO suppressed HIF-1 α under 1% O_2 conditions

By using siRNA, we demonstrated that a decreased level of MnSOD resulted in a significant increase in the steady-state level of $O_2^{\bullet-}$ and an induction of HIF-1 α protein. To determine whether $O_2^{\bullet-}$ is an important molecular species responsible for the induction of HIF-1 α during hypoxia levels of $O_2^{\bullet-}$ were lowered in MCF-7 cells with the spin trapping agents POBN or DMPO. Different concentrations of POBN or DMPO (33–100 mM) were introduced to the cells under both 21% and 1% O_2 conditions and HIF-1 α protein was determined. These high concentrations are necessary because of their low rate of reaction with $O_2^{\bullet-}$ compared to the naturally occurring SODs. POBN was present through the 4-h incubation. However, because of its high reactivity and propensity to form oxidation products, DMPO was added only for the final hour of the hypoxic incubation. As expected, HIF-1 α protein was detectable only under 1% O_2 . Cells treated with these spin traps had decreased levels of HIF-1 α following the hypoxic incubation. HIF-1 α protein induction appeared to be decreased in a spin trap concentration-dependent manner (Fig 3A).

To monitor other consequences of introducing spin trapping agents during the hypoxic incubation, the ability of cells to produce ROS after the hypoxic incubation was examined. Relative levels of ROS were assessed under both normoxia and hypoxia. Media was removed and fresh DMPO (100 mM) in chelated-PBS was introduced. The signal heights from the EPR spectra normalized to the amount of protein was lower from those cells exposed to spin trap (100 mM) during the hypoxic incubation (Fig 3B). These results are consistent with $O_2^{\bullet-}$ being involved in HIF-1 α induction under hypoxia.

Scavenging of $O_2^{\bullet-}$ by Tempol affected HIF-1 α protein induction after exposure to 1% O_2

Data from the spin trapping experiments suggested a role for $O_2^{\bullet-}$ in the regulation of the hypoxic accumulation of HIF-1 α . However, spin traps do not mimic SOD activity because they do not produce H_2O_2 as a product upon their reaction with $O_2^{\bullet-}$. In another approach to test our hypothesis we used Tempol as an $O_2^{\bullet-}$ scavenger. Tempol is a stable nitroxide radical that has SOD mimetic activity [23]; it dismutates two $O_2^{\bullet-}$ radicals producing H_2O_2 and O_2 . When concentration is sufficiently high, it has been reported that Tempol will react with the protonated form of $O_2^{\bullet-}$ (hydroperoxyl radical, $^{\bullet}OOH$) to produce H_2O_2 and oxoammonium salts [24]. Cells were treated with various concentrations of Tempol (0.1 - 40 mM) under hypoxic conditions. Tempol induced HIF-1 α accumulation under hypoxic conditions as the concentration increased (Fig 4A). This effect appeared to be dose-dependent since at 10 mM Tempol, a decrease in HIF-1 α protein was observed; a further decrease was seen with 20 mM and HIF-1 α was undetectable at 40 mM. The level of MnSOD protein was not affected. Following hypoxia, the ability of cells to generate DMPO-OH signal was determined. Cells treated with 0.1 or 1 mM Tempol generated significantly less DMPO-OH relative to untreated controls at 1% O_2 ($p < 0.05$). When higher concentrations of Tempol (10, 20, and 40 mM) were used, the signal for DMPO-OH was not visible because the peaks were masked by the Tempol signal (Fig 4B). These results suggest that the level of $O_2^{\bullet-}$ generated by cells treated with Tempol was significantly decreased relative to untreated cells at 1% O_2 . Extracellular levels of H_2O_2 were not altered at lower concentrations of Tempol (0.1 to 10 mM), but increased with 20 mM and 40 mM relative to untreated cells at 1% O_2 ($p < 0.05$), Fig 4C. These observations are consistent with $O_2^{\bullet-}$ being an important molecular effector underlying hypoxic HIF-1 α stabilization.

SOD mimic suppressed HIF-1 α induction under 1% O₂

To further analyze the effects of ROS removal and/or ROS generation in MCF-7 cells, we used the AEOL10113, a small molecular weight manganese-containing porphyrin that has potent SOD mimic activity [25]. It has been reported that AEOL10113 can reduce hypoxia-induced O₂^{•−} levels and VEGF production by macrophages [26]. Different concentrations of the compound were introduced to MCF-7 cells at 21% O₂ for 4 h, then cells were treated with hypoxia and HIF-1 α protein was determined. At a low concentration of AEOL10113 (10 μ M), HIF- α was found to be decreased; an additional decrease was seen with 20 μ M mimetic. However, when AEOL10113 concentrations were increased further, HIF-1 α levels increased (Fig 5A). The changes in the fold-intensities were measured by IDV. Consistent with its SOD mimetic activity, the level of O₂^{•−} decreased (Fig 5B) and the level of H₂O₂ increased [27] (Fig 5C). Interestingly, at 50 μ M AEOL10113, a concentration where O₂^{•−} was lowest relative to untreated cells ($p < 0.05$), the accumulation of H₂O₂ was greatest. This biphasic response parallels to that observed by Wang *et al.* [16].

Changes in superoxide levels affected clonogenic survival of MCF-7 cells

In spin trapping and O₂^{•−} scavenger experiments, we observed a significant decrease in DMPO-OH signals, which implies that O₂^{•−} levels in the cells had been altered. We hypothesized that the changes in O₂^{•−} levels induced by these spin traps and O₂^{•−} scavengers may be harmful and thus contribute to cell mortality. To test the hypothesis, we evaluated the cytotoxicity of these agents by determining clonogenic survival of the cells after treatment during the exposure to hypoxia. Cells treated with either POBN or DMPO during 1% O₂-exposure showed no significant difference in survival fractions relative to untreated control cells ($p > 0.05$), but the surviving fractions were significantly decreased relative to untreated cells at 21% O₂ ($p < 0.01$), Fig 6A, B. Cells treated with either 1% O₂ or 1% O₂ + Tempol showed a significant decrease in survival fraction ($p < 0.01$) relative to untreated cells at 21% O₂; cells treated with 1% O₂ + Tempol exposure showed a significant decrease in survival fraction relative to control 1% ($p < 0.01$) only when the concentrations of Tempol were higher than 10 mM (Fig 6C). However, cells treated with AEOL10113 at 1% O₂ conditions showed no significant difference in survival fraction compared to untreated cells at 21% and 1% O₂; only cells treated with 1% O₂ + AEOL10113 at 200 μ M exposure showed a significant decrease in survival fraction relative to 1% O₂ + AEOL10113 20 μ M ($p < 0.05$) (Fig 6D).

DISCUSSION

In recent years, much effort has been devoted to the use of respiratory inhibitors or ρ^0 cells to examine the role of the mitochondrial electron transport chain in the regulation of HIF-1 α . It is likely that mechanisms of O₂ sensing and signaling during hypoxia are associated with mitochondrial ROS generation and involves different pathways in different cell types [10,17, 28]. Work using genetic approaches has demonstrated that HEK293 cells transfected with siRNA against the Rieske iron-sulfur protein of mitochondria complex III failed to stabilize HIF-1 α protein during hypoxia. In addition, both wild-type human fibroblasts and cells that had an impairment of oxidative phosphorylation exhibited an increase in HIF-1 α protein stabilization when exposed to hypoxia (1.5% O₂), which was prevented by the addition of myxothiazol. The authors concluded that oxidative phosphorylation is not required for the hypoxic stabilization of HIF-1 α , but mitochondrial ROS are needed [17]. Therefore, ROS play a major role in stabilizing HIF-1 α .

The exact molecular nature of the ROS responsible for regulation of HIF-1 α under hypoxia is not clear. Wang *et al.* found that overexpression of MnSOD resulted in a biphasic effect on HIF-1 α protein levels [16]. They demonstrated that with relatively low overexpression of MnSOD, HIF-1 α decreased. Because an increase in MnSOD would lower the steady-state level

of superoxide, this observation suggests that superoxide may play a role in the stabilization of HIF-1 α protein. However, when MnSOD was highly overexpressed, HIF-1 α was again present. Because high levels of MnSOD can lead to greater fluxes of H₂O₂, this suggests that H₂O₂ may also regulate HIF-1 α . Goyal *et al.* found that overexpression of a NADPH oxidase 1 (Nox1), which generates high fluxes of O₂^{•-}, in human lung adenocarcinoma A549 cells resulted in accumulation of HIF-1 α in normoxia [29]; under hypoxia (1% O₂), an additional increase was observed. These effects could be reversed by the flavoprotein inhibitor diphenylene iodonium or by catalase. These observations are consistent with high levels of H₂O₂ being able to activate HIF-1 α . Therefore the appearance of HIF-1 α at higher levels of MnSOD as well as with the activation of Nox1 suggests that high levels of H₂O₂ can lead to accumulation of HIF-1 α . To address these possible roles of ROS in stabilizing HIF-1 α , we carried out experiments using ROS scavengers.

When MCF-7 cells were exposed to nontoxic concentrations of Tempol (< 10 mM), the protein level of HIF-1 α increased, Fig 4A. Concomitantly, the level of O₂^{•-}, as seen by the intensity of the DMPO-OH signal, was decreased, Fig 4B. At nontoxic concentrations of Tempol, there was no change in the level of H₂O₂, Fig 4C. (Concentrations of Tempol (> 1 mM) are toxic, as seen by decreased cell survival Fig 6C.) Tempol is a redox active compound and may well intercept the ferryl state of PHD, not allowing the hydroxylation of HIF-1 α to occur. That Tempol allows HIF-1 α to accumulate may explain the many positive *in vivo* observations reported with this compound (30). Thus, firm conclusions on the identity of a specific ROS that regulates HIF-1 α can not be made from these observations. To better probe for the identity of the ROS we carried out experiments with an SOD mimic, AEOL10113 [24]. This SOD mimic had no effect on cell survival, Fig 6D.

To address better the role of ROS in the modulation of HIF-1 α , we introduced varying levels of AEOL10113 to cells in combination with exposure to hypoxia. Similar to the observations of Wang *et al.* [16], we observed a biphasic effect in the accumulation of HIF-1 α with varying concentration of AEOL10113. At low concentrations of SOD mimic, we observed decreased levels of HIF-1 α in MCF-7 cells and a concomitant decrease in the levels of O₂^{•-} during hypoxia as studied by EPR spin trapping, Fig 5A and B. Whereas at higher concentrations of SOD mimic, corresponding increases in H₂O₂ were seen with parallel increases in HIF-1 α protein. Because the SOD mimic altered the levels of both O₂^{•-} and H₂O₂ the precise roles of O₂^{•-} and H₂O₂ cannot be deconvoluted. Therefore, we took another approach to alter the endogenous levels of O₂^{•-} and MnSOD.

In the experiments with Tempol and AEOL10113, the goal was to increase the effective SOD-like activity in cells. To specifically decrease the endogenous MnSOD-activity, we used siRNA against MnSOD. This should result in an increase in the steady-state level of O₂^{•-}, which should lead to an increase in HIF-1 α . Indeed, upon introduction of siRNA we observed the anticipated lowering of MnSOD, and an increase in both O₂^{•-} and HIF-1 α , Fig 2A and C. There was no detectable change in the level of H₂O₂, Fig 2D. These observations point directly to O₂^{•-} as a modulator of HIF-1 α stabilization during hypoxia.

To provide additional evidence for the role of O₂^{•-} in modulating HIF-1 α , we used the spin traps POBN and DMPO as scavengers of O₂^{•-}. Lowering the steady-state level of O₂^{•-} should lower HIF-1 α under hypoxia. We found that both POBN and DMPO lowered the level of HIF-1 α protein, Fig 3A. Neither POBN nor DMPO were significantly toxic to the cells under our experimental conditions, Fig 6A and B. Taking all observations together, it is clear that O₂^{•-} has a major role in regulating HIF-1 α .

Here, we propose that MnSOD plays an important role in regulating HIF-1 α accumulation during hypoxia by modulating the levels of O₂^{•-}. To our knowledge, this is the first

demonstration that MnSOD regulates HIF-1 α via O₂^{•-}. These results should provide a better understanding of the biological role of MnSOD in regulating HIF-1 α in tumor cells.

Acknowledgements

The authors thank Dr. James D. Crapo, Department of Medicine, National Jewish Medical and Research Center, Denver, CO, who provided the SOD mimic; Dr. Rebecca E. Oberley for her help; Dr. Melissa L.T. Teoh for technical help with siRNA experiments; Dr. Douglas R. Spitz for technical advice on MnSOD activity assays, and Dr. Terry D. Oberley, University of Wisconsin School of Medicine and Public Health, Madison, WI, for his help in preparing the manuscript. This work was funded in part by the Milheim Foundation 2005-16. SK was partially supported by the Ministry of Science under the Royal Thai Government and the Free Radical and Radiation Biology Program of The University of Iowa.

References

1. Semenza GL. Regulation of mammalian O₂ homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol* 1999;15:551–78. [PubMed: 10611972]
2. Wenger R. Mammalian oxygen sensing, signalling and gene regulation. *J Exp Biol* 2000;203:1253–63. [PubMed: 10729275]
3. Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem* 1995a;270:1230–37. [PubMed: 7836384]
4. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci USA* 1995b;92:5510–14. [PubMed: 7539918]
5. Jaakkola P, Mole DR, Tian YM, et al. Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science* 2001;292:468–72. [PubMed: 11292861]
6. Kallio P, Wilson W, O'Brien S, Makino Y, Poellinger L. Regulation of the hypoxia-inducible transcription factor 1 α by the ubiquitin-proteasome pathway. *J Biol Chem* 1999;274:6519–25. [PubMed: 10037745]
7. Tanimoto K, Makino Y, Pereira T, Poellinger L. Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the Von Hippel-Lindau tumor suppressor protein. *EMBO J* 2000;19:4298–4309. [PubMed: 10944113]
8. Nilsson I, Shibuya M, Wennstrom S. Differential activation of vascular genes by hypoxia in primary endothelial cells. *Exp Cell Res* 2004;299:476–85. [PubMed: 15350545]
9. Oberley LW, Buettner GR. Role of superoxide dismutase in cancer: a review. *Cancer Res* 1979;39:1141–49. [PubMed: 217531]
10. Chandel NS, McClintock DS, Feliciano CE, et al. Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1 α during hypoxia: a mechanism of O₂ sensing. *J Biol Chem* 2000;275:25130–38. [PubMed: 10833514]
11. Schroedl C, McClintock DS, Budinger GR, Chandel NS. Hypoxic but not anoxic stabilization of HIF-1 α requires mitochondrial reactive oxygen species. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L922–31. [PubMed: 12376345]
12. Park JH, Kim TY, Jong HS, et al. Gastric epithelial reactive oxygen species prevent normoxic degradation of hypoxia-inducible factor-1 α in gastric cancer cells. *Clin Cancer Res* 2003;9:433–440. [PubMed: 12538497]
13. Yang LY, Chen WL, Lin JW, et al. Differential expression of antioxidant enzymes in various hepatocellular carcinoma cell lines. *J Cell Biochem* 2005;96:622–31. [PubMed: 16052474]
14. Soini Y, Vakkala M, Kahlos K, Paakko P, Kinnula V. MnSOD expression is less frequent in tumour cells of invasive breast carcinomas than in *in situ* carcinomas or non-neoplastic breast epithelial cells. *J Pathol* 2001;195:156–62. [PubMed: 11592093]
15. Zhang H, Yan T, Oberley TD, Oberley LW. Comparison of effect of two polymorphic variants of manganese superoxide dismutase on human breast MCF-7 cancer cell phenotype. *Cancer Res* 1999;59:6276–83. [PubMed: 10626823]

16. Wang M, Kirk J, Venkataraman S, et al. Manganese superoxide dismutase suppresses hypoxic induction of hypoxia-inducible factor-1 α and vascular endothelial growth factor. *Oncogene* 2005;24:8154–66. [PubMed: 16170370]
17. Brunelle J, Bell E, Quesada N, et al. Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. *Cell metabolism* 2005;1:409–14. [PubMed: 16054090]
18. Beauchamp C, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 1971;44:276–87. [PubMed: 4943714]
19. Sun Y, Elwell JH, Oberley LW. A simultaneous visualization of the antioxidant enzymes glutathione peroxidase and catalase on polyacrylamide gels. *Free Rad Res Commun* 1988;5:67–75.
20. Oberley, LW.; Spitz, DR. *CRC Handbook of Methods for Oxygen Radical Research* Greenwald Ra. Boca Raton, FL: CRC Press; 1985. p. 217–20.
21. Buettner GR. In the absence of catalytic metals, ascorbate does not autoxidize at pH 7: Ascorbate as a test for catalytic metals. *J Biochem Biophys Meth* 1988;16:27–40. [PubMed: 3135299]
22. Panus PC, Radi R, Chumley PH, Lillard RH, Freeman BA. Detection of H₂O₂ release from vascular endothelial cells. *Free Radic Biol Med* 1993;14:217–23. [PubMed: 8425723]
23. Samuni A, Krishna CM, Riesz P, Finkelstein E, Russo A. A novel metal-free low molecular weight superoxide dismutase mimic. *J Biol Chem* 1998;263:17921–24. [PubMed: 2848018]
24. Batinic-Haberle I, Benov L, Spasojevic I, Fridovich I. The *ortho* effect makes manganese (III) meso-tetrakis N-methylpyridinium a powerful and potentially useful superoxide dismutase mimic. *J Biol Chem* 1998;273:24521–28. [PubMed: 9733746]
25. Krishna MC, Grahame DA, Samuni A, Mitchell JB, Russo A. Oxoammonium cation intermediate in the nitroxide-catalyzed dismutation of superoxide. *Proc Natl Acad Sci USA* 1992;89:5537–41. [PubMed: 1319064]
26. Jackson IL, Chen L, Batinic-Haberle I, Vujaskovic Z. Superoxide dismutase mimetic reduces hypoxia-induced O₂^{•−}, TGF-beta, and VEGF production by macrophages. *Free Radic Res* 2007;41:8–14. [PubMed: 17164174]
27. Buettner GR, Ng CF, Wang W, Rodgers VGJ, Schafer FQ. A new paradigm: Manganese superoxide dismutase influences the production of H₂O₂ in cells and thereby their biological state. *Free Radic Biol Med* 2006;41:1338–50. [PubMed: 17015180]
28. Emerling BM, Plataniias LC, Black E, Nebreda AR, Davis RJ, Chandel NS. Mitochondrial reactive oxygen species activation of p38 mitogen-activated protein kinase is required for hypoxia signaling. *Mol Cell Biol* 2005;25:4853–62. [PubMed: 15923604]
29. Goyal P, Weissmann N, Grimminger F, et al. Upregulation of NAD(P)H oxidase 1 in hypoxia activates hypoxia-inducible factor 1 *via* increase in reactive oxygen species. *Free Radic Biol Med* 2004;36:1279–88. [PubMed: 15110393]
30. Soule BP, Hyodo F, Matsumoto K-I, Simone NL, Cook JA, Krishna MC, Mitchell JB. The chemistry and biology of nitroxide compounds. *Free Radic Biol Med* 42:1632–1650. [PubMed: 17462532]

Figure 1. Transient siRNA knockdown of MnSOD

A) Western blot analysis of whole cell lysates showing MnSOD protein levels of immortalized non-malignant breast cells, MCF-10A and human breast adenocarcinoma, MCF-7. **B and C)** MCF-7 cells were transfected with MnSOD siRNA and nontargeting siRNA (siNeg) at different times and concentrations. Whole cell lysates were analyzed by western blot for MnSOD expression using actin as a protein loading control. MnSOD protein expression was compared relative to untransfected (control) or nontargeting siRNA (siNeg). **D)** MnSOD activities were determined in MCF-7 cells transfected with 300 pmol siRNA for 72 h by nondissociating electrophoresis (12% gels) stained for SOD activity. All results are representative of at least three separate experiments.

Figure 2. Inhibition of MnSOD in MCF-7 cells by siRNA significantly increased O₂^{•-} levels and an induction of HIF-1 α protein under 1% O₂ conditions

A) Cells were transfected with siRNA against MnSOD or siNeg as a non-targeting control (300 pmol, 72 h) or exposed to transfection reagent alone (control). Cells were exposed to hypoxia (1% O₂) for 4 h. Whole cell lysates were analyzed for the expression of MnSOD and HIF-1 α protein by western blot analysis. Relative band intensities for HIF-1 α are presented under the blots. **B)** Representative EPR spectra, measured at 21% and 1% O₂, showing the DMPO-OH spin adduct ($a^N = a^H = 14.9$ G) normalized to the protein. The intensity of the DMPO-OH signal corresponds to the relative rate of O₂^{•-} formation. **C)** The EPR peak height (normalized to the amount of protein) measured from cells transfected with siRNA against MnSOD is

significantly different from siNeg and control, *i.e.* untransfected cells, $p < 0.01$. **D)** Extracellular H_2O_2 accumulation was determined by *p*HPA fluorescence assay, $p > 0.05$ relative to siNeg. All results are representative of at least three separate experiments.

Figure 3. Scavenging of O₂^{•−} by spin traps POBN or DMPO suppressed HIF-1α protein under 1% O₂ conditions

A) MCF-7 cells were incubated in the absence (0 mM) and presence (33–100 mM) of POBN under 21% or 1% O₂ for 4 h. For the DMPO experiments the spin trap was added after cells were pre-treated with 1% O₂ for 3 h; then the incubation was continued for 1 h under 1% O₂. Whole cell lysates were analyzed for expression of HIF-1α protein by western blot analysis.

B) The EPR peak height of the DMPO-OH spin adduct normalized to the amount of protein was significantly decreased from cells treated with DMPO (100 mM at both 21% and 1% O₂) during the hypoxic incubation relative to untreated control. For these experiments, the media with spin trap for the hypoxic incubation was removed from the cells and replaced with chelated-PBS containing fresh DMPO (100 mM). Spectra were collected as given in methods.

Figure 4. Scavenging of O₂^{•−} by Tempol affected HIF-1 α protein accumulation under 1% O₂ conditions

A) MCF-7 cells were treated with Tempol at different concentrations (0.1 – 40 mM) at 1% O₂ for 4 h. Whole cell lysates were analyzed for the expression of HIF-1 α and MnSOD protein by western blot analysis. **B)** EPR signal height of DMPO-OH normalized to the amount of protein obtained from cells treated with various concentration of Tempol, $p < 0.05$ relative to 1% O₂ control. # The DMPO-OH adduct peaks in the presence of 10 – 40 mM Tempol were masked by the Tempol EPR signal and thus could not be quantified. **C)** Extracellular H₂O₂ formation was determined in Tempol-treated cells by the *p*HPA fluorescence assay, $p < 0.05$ relative to 1% control.

Figure 5. SOD mimic suppressed HIF-1 α expression under 1% O₂ conditions

A) Different concentrations (10 – 200 μM) of AEOL10113 were added to MCF-7 cells at 21% O₂ for 4 h followed by incubation at 1% O₂ for 4 h. HIF-1 α protein expression was analyzed from whole cell lysates by western blot. Relative band intensities for HIF-1 α are presented under the blots. **B)** Quantified data from the EPR spectra normalized to the amount of protein obtained from cells treated with different concentrations of AEOL10113, $p < 0.05$ relative to 1% control. **C)** Extracellular H₂O₂ accumulation was determined by the *p*HPA fluorescence assay, $p < 0.05$ relative to 1% untreated control.

Figure 6. Clonogenic survival after treatment with superoxide-removal by spin traps, O₂^{•-} scavenger, and SOD mimic

A) MCF-7 cells were treated with 100 mM POBN under both 21% and 1% O₂ for 4 h. **B)** DMPO 100 mM was added after cells were pre-treated with 1% O₂ for 3 h then the incubation was continued for 1 h under 1% O₂. $p < 0.01$ compared to 21% control and $p > 0.05$ compared to 1% control. **C)** Cells were treated with different concentrations of Tempol at 1% O₂, $p < 0.01$ compared to 21% and 1% O₂ control respectively. **D)** Cells were pre-treated with AEOL10113 at 21% O₂ for 4 h then 1% O₂ incubation was continued for 4 h. $p > 0.05$ compared to 21% and 1% O₂ control and $p < 0.05$ compared to 1% O₂ + 20 μM AEOL10113. To examine

the effect of $O_2^{\bullet-}$ removal under hypoxia on cell proliferation clonogenic assays were performed as in Methods section.