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Manganese superoxide dismutase suppresses hypoxic induction of hypoxia-inducible factor-1α and vascular endothelial growth factor

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Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that governs cellular responses to reduced O_2 availability by mediating crucial homeostatic processes. HIF-1 is composed of an HIF-1α subunit and an HIF-1β subunit. HIF-1α is degraded following enzyme-dependent hydroxylation of prolines of HIF-1α in the presence of molecular oxygen, Fe^{2+}, α-ketoglutarate, and ascorbate. These cofactors contribute to the redox environment of cells. The antioxidant enzyme manganese superoxide dismutase (MnSOD) also modulates the cellular redox environment. Here we show that MnSOD suppressed hypoxic accumulation of HIF-1α protein in human breast carcinoma MCF-7 cells. This suppression was biphasic depending on MnSOD activity. At low levels of MnSOD activity, HIF-1α protein accumulated under hypoxic conditions. At moderate levels of MnSOD activity (two- to six-fold increase compared to parent cells), these accumulations were blocked. However, at higher levels of MnSOD activity (>6-fold increase), accumulation of HIF-1α protein was again observed. This biphasic modulation was observed under both 1 and 4% O_2. Coexpression of mitochondrial hydrogen peroxide-removing proteins prevented the accumulation of HIF-1α protein in cells with high levels of MnSOD; this effect demonstrates that the restabilization of HIF-1α observed in high MnSOD overexpressors is probably due to hydrogen peroxide, most likely produced from MnSOD. Hypoxic induction of vascular endothelial growth factor (VEGF) protein was also suppressed by elevated MnSOD activity and its levels reflected HIF-1α protein levels. These observations demonstrated that HIF-1α accumulation and VEGF expression could be modulated by the antioxidant enzyme MnSOD.
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Introduction

Hypoxia describes a low oxygen concentration environment that can be caused by stroke, coronary artery disease, trauma, or high rate of cell proliferation such as encountered in solid tumors (Dewhirst *et al*., 1989). Because the proliferation rate of tumor cells is usually faster than that of normal endothelial cells that form microvessels into tumors, tumor microvessels do not function as efficiently as those in normal tissues, leading to the widespread hypoxia in solid tumors (Shah-Yukich and Nelson, 1988).

Tumor cells that have adapted to hypoxic conditions are thought to play critical roles in tumor progression (Hockel *et al*., 1996). Among the first responses at the onset of hypoxia is an increase in the protein levels of hypoxia-inducible factor-1 (HIF-1) (Wang and Semenza, 1993b). HIF-1 responds to reduced O_2 availability by mediating crucial homeostatic processes such as angiogenesis, glycolysis, and erythropoiesis (Semenza, 2000). As a transcription factor, HIF-1 has more than 40 target genes and the number continues to increase. Vascular endothelial growth factor (VEGF) is one of HIF-1 downstream genes (Liu *et al*., 1995). VEGF is a powerful mitogenic cytokine specific for endothelial cells; its production triggers the angiogenic cascade and tumor neovascularization processes (Nagy *et al*., 2002). VEGF controls not only the onset, but also the extent and duration of these processes. Hypoxia, which is a common characteristic of solid tumors, is a stimulus leading to the induction of VEGF via the regulation of HIF-1 (Semenza, 2001b; Kim *et al*., 2003; Pugh and Ratcliffe, 2003a).

HIF-1 is a heterodimer consisting of two subunits, HIF-1α and HIF-1β (Wang and Semenza, 1993a). When oxygen is sufficient, HIF-1α is constantly being made and then degraded by the ubiquitin–proteasome pathway via the von Hippel–Lindau (VHL) tumor suppressor protein (Maxwell *et al*., 1999). VHL protein binds to HIF-1α, causing ubiquitinylation of this protein, which is then degraded by the 26S proteasome (Salceda and Caro, 1997). The binding of VHL to HIF-1α requires hydroxylation of proline residues inside the oxygen-dependent degradation domain (ODD) of HIF-1α (Bruick and McKnight, 2001). Proline[402] and proline[564]

are hydroxylated by HIF-prolyl hydroxylase, which needs the presence of several cofactors to gain full activity. These cofactors are molecular oxygen, Fe^{2+}, α-ketoglutarate, and ascorbate (Jaakkola *et al*., 2001; Kondo and Kaelin, 2001; Semenza, 2001a; Pugh and Ratcliffe, 2003b). During hydroxylation, one atom of the O_2 molecule is incorporated into succinate upon decarboxylation of α-ketoglutarate and the other into a hydroxyl group on the proline residue. The Fe^{2+} is located inside the active site of HIF-prolyl hydroxylase (McNeill *et al*., 2002).

Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide (H_2O_2), are known to serve as signal transducers (Nakamura *et al*., 1997; Powis *et al*., 1997; Suzuki *et al*., 1997; Schafer and Buettner, 2001). It has been shown that ROS can participate in the hypoxia signal transduction pathway that mediates the stabilization of HIF-1α (Chandel *et al*., 2000; Schroedl *et al*., 2002; Park *et al*., 2003). Manganese superoxide dismutase (MnSOD) is a primary antioxidant enzyme that is located in the mitochondrial matrix. It has been shown that the malignant phenotype is suppressed when MnSOD activity is elevated in certain cancer cells (Li *et al*., 1998a, b; Zhang *et al*., 1999). MnSOD has been implicated as a tumor suppressor and as a metastasis suppressor in some tumor cell lines (Oberley and Buettner, 1979; Oberley and Oberley, 1988; Bravard *et al*., 1992). MnSOD modulates the cellular redox environment by converting superoxide radical ($O_2^{\cdot-}$) to H_2O_2 and dioxygen. Therefore, MnSOD may affect the expression of redox-sensitive genes, including HIF-1. Here we are the first to show that MnSOD overexpression suppressed the hypoxic accumulation of HIF-1α in human breast carcinoma MCF-7 cells. The hypoxic induction of VEGF protein was also suppressed by elevation of MnSOD.

Results

Increased MnSOD activity suppressed hypoxic accumulation of HIF-1α protein in cells exposed to 1% O_2

To test the role of elevated MnSOD activity on hypoxic accumulation of HIF-1α protein, 12 previously characterized (Zhang *et al*., 1999) stably transfected MCF-7 clones with different MnSOD activities as well as parental (untransfected) cells were used. These clones were produced by transfection with pcDNA3 plasmids containing sense MnSOD cDNA or containing no MnSOD insert (Neo clone) by the lipofectAmine (Life Technologies, Gaithersburg, MD, USA) method. The G418-resistant colonies were isolated by cloning rings and maintained in medium supplemented with 400 μg/ml G418 (Life Technologies). The MnSOD clones were characterized and found to have a less malignant phenotype than the parental cells as indicated by (1) decreased plating efficiency, (2) elongated cell population doubling time, (3) lower clonogenic fraction in soft agar, and (4) complete inhibition or delayed onset of tumor formation in nude mice, as well as slower growth of tumors that formed (Zhang *et al*., 1999). These 13 different cell lines were exposed to 1% O_2 for 4 h. This condition did not change MnSOD activity during this time frame (data not shown). Immediately after hypoxia, protein was harvested and HIF-1α protein levels were analysed by Western blotting (Figure 1a). The results showed that at low levels of MnSOD activity (parental cells (WT) and transfected clones with the same activity (Neo and SOD23)), HIF-1α protein accumulated. In clones where MnSOD activity was moderately increased (two- to six-fold relative to that of the parent cells), HIF-1α protein levels were decreased. The levels of HIF-1α protein decreased with increasing MnSOD activity until they were no longer detectable in clones with three- and six-fold increase in MnSOD activity. However, in clones where MnSOD activity was increased to a higher level (>6-fold), HIF-1α protein was again detected, and increased with increasing MnSOD activity. Regression analysis demonstrated a linear inverse correlation of HIF-1α with low levels of MnSOD activity (one- to three-fold), whereas at high levels of MnSOD (>6-fold), a linear positive correlation was observed (Figure 1b).

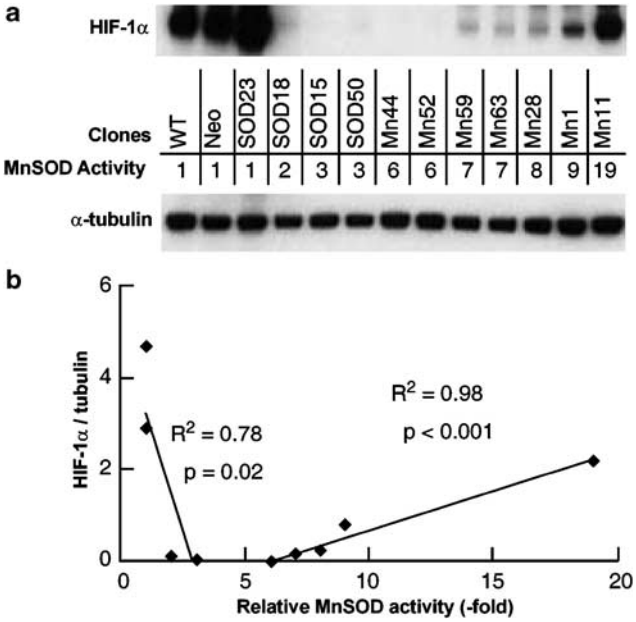

Figure 1 Plasmid transfection of MnSOD suppressed HIF-1α protein accumulation after 4 h exposure to 1% O_2. (**a**) Western blot showing HIF-1α protein levels in 12 different MnSOD stably transfected clones plus parental MCF-7 cells after 4 h exposure to 1% O_2. The first three lanes were samples from MCF-7 parental cells, Neo vector control, and a clone with the same activity as parental cells. The remaining 10 lanes were samples from clones with increasing MnSOD activity. The bottom row of numbers indicates the relative MnSOD activity compared to the parental MCF-7 cells. The actual MnSOD activities of the clones were previously measured (Zhang *et al*., 1999) to be (in units/mg protein) WT (7±2), Neo4 (7±3), SOD15 (19±4), SOD18 (11±2), SOD23 (10±2), SOD50 (23±6), Mn1 (62±8), Mn11 (142±20), Mn28 (57±10), Mn44 (44±9), Mn52 (44±6), Mn59 (53±2), and Mn63 (52±8). (**b**) Regression analysis of HIF-1α protein and MnSOD activity shows a biphasic effect. HIF-1α protein levels were quantified by densitometry and normalized to α-tubulin levels. Similar results were obtained in a repeated experiment

In order to confirm and extend this observation with another technique besides the stable transfection approach, MCF-7 cells were transduced with adenovirus-containing MnSOD cDNA. This transduction increased the enzymatic activity of MnSOD as determined by activity gel analyses; MnSOD activity increased with increasing multiplicity of infection (MOI), which is calculated from plaque forming units (PFU; Figure 2a). The increase in MnSOD activity with increasing MOI was confirmed with a spectrophotometric enzymatic activity assay (data not shown). The transduction efficiency after adenoviral infection using MnSOD immunofluorescence was also measured. A small percentage of the cells were positive for MnSOD in the control cells (Table 1) and the percentage of cells that showed fluorescence increased with increasing MOI. At the maximum MOI used (200), 73% of the cells demonstrated immunofluorescent MnSOD protein. Following adenovirus transduction in 21% O_2, cells were exposed to 1% O_2 for 4 h and samples were then collected. SOD activity (activity gel assay) and HIF-1α protein levels (Western blotting) were assessed. In MCF-7 parental cells, HIF-1α protein accumulated after hypoxia exposure; increased MnSOD activity suppressed the accumulation of HIF-1α protein under 1% O_2 (Figure 2b). HIF-1α protein levels dropped significantly starting at 5 MOI of adenoviral MnSOD and became undetectable at 50 MOI. HIF-1α protein was again detectable at 75 MOI and increased with increasing MOI thereafter, but not reaching the level found in parental cells. This biphasic effect is consistent with what we observed with MCF-7 clones that were stably transfected with MnSOD cDNA (Figure 1). In both plasmid transfection and adenovirus transduction experiments, HIF-1α protein was undetectable in cells grown at 21% O_2 (data not shown).

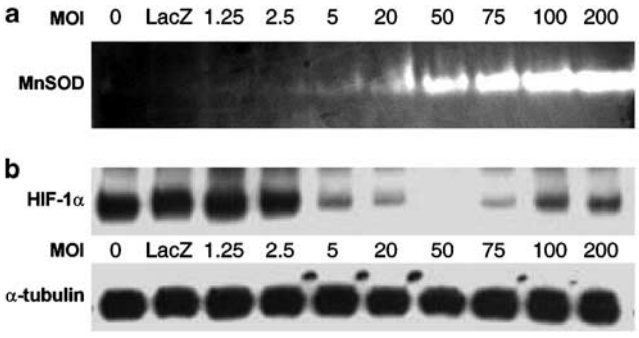

Figure 2 Adenoviral transduction of MnSOD suppressed HIF-1α protein accumulation after 4 h exposure to 1% O_2. (**a**) MnSOD activity gel analysis showed that MnSOD activity increased with increasing MOI (from 1.25 up to 200). Adenoviral transduction was performed at 21% O_2 and the transduced cells were exposed to 1% O_2 for 4 h. Samples labeled LacZ were from cells transduced with Ad*LacZ*. (**b**) Western blots showed HIF-1α protein levels in Ad*MnSOD*-transduced cells exposed to 1% O_2 for 4 h. α-Tubulin was used as an internal control. Primary antibodies to HIF-1α and α-tubulin were mixed together to probe the blot. Similar results were obtained in a repeated experiment

Table 1 Ad*MnSOD* transduction increased immunofluorescence for MnSOD protein in MCF-7 cells to 70% with 200 MOI (500 cells from three to four fields were counted)

MOI	*Cells positive (%)*
0	9
Ad*LacZ* (200 MOI)	2
1.25	22
2.5	25
5	23
20	36
50	47
75	45
100	58
200	73

Increased MnSOD activity suppressed hypoxia-induced accumulation of HIF-1α protein in cells exposed to 4% O_2

The oxygen concentration inside a large solid tumor is heterogeneous. Some regions have very low pO_2 values while others have pO_2 values near to that of normal tissues (Moulder and Martin, 1984; Collingridge *et al*., 1997). Because MnSOD suppressed HIF-1α protein accumulation in MCF-7 cells exposed to 1% O_2, both with adenoviral transduction and plasmid transfection, it was further investigated if this suppressive effect existed when cells were exposed to 4% O_2. This is a value typical of normal tissue oxygen concentration (Jiang *et al*., 1996) but still lower than the 21% O_2 usually used for most cell culture conditions. Using the same stable MnSOD-overexpressing clones described above, it was found that HIF-1α protein began to decrease in clones that expressed a two-fold increase in MnSOD activity and remained suppressed in clones that had up to a six-fold increase in MnSOD activity. However, when MnSOD activity was increased higher than eight-fold, HIF-1α levels were again increased (Figure 3a). In order to examine this with another method, MCF-7 cells transduced at 21% O_2 with increasing MOI of adenoviral MnSOD were then exposed to 4% O_2. As MnSOD activity increased with increasing adenovirus titer from that of parental MCF-7 cells, HIF-1α levels decreased, reaching a minimum at 20 and 50 MOI (Figure 3b). At 75 MOI, HIF-1α levels increased slightly and continued to increase with increasing MOI, but again not reaching the level of parental cells. The results observed at 4% O_2 parallel those of 1% O_2, suggesting that the suppressive effect of MnSOD on HIF-1α is common at both oxygen concentrations.

Hypoxia (1% O_2) induced VEGF mRNA expression in a time-dependent manner in MCF-7 cells

Angiogenesis is a key step that not only supports tumor growth with nutrients and oxygen, but also provides a ready route for cells to escape the primary site, leading to metastases (Brown *et al*., 1997). VEGF is a potent angiogenic mitogen that mediates the angiogenesis process (Dvorak *et al*., 1995a; Nagy *et al*., 2002). Exposure to hypoxia stimulates the production of

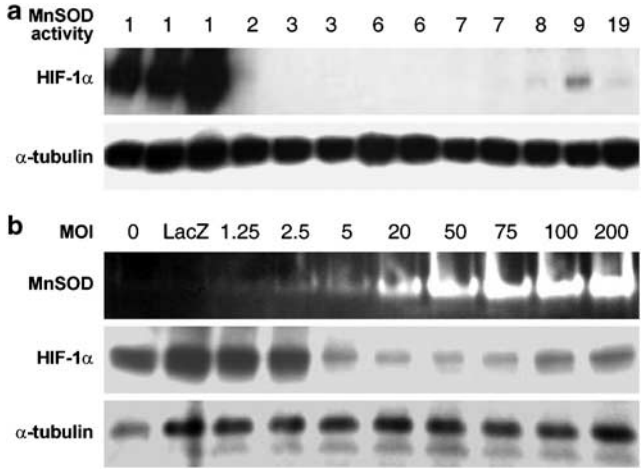

Figure 3 Elevated MnSOD activities suppressed HIF-1α protein accumulation at 4% O_2. (**a**) Plasmid transfection of MnSOD suppressed normoxic (4% O_2, 4 h) accumulation of HIF-1α protein. Western blot shows HIF-1α protein levels in different MnSOD stably transfected clones of MCF-7 cells with 4 h of exposure to 4% O_2. The top row of numbers indicates the relative MnSOD activity in 12 different clones compared to MCF-7 WT cells. α-Tubulin was used as an internal control. The 12 different clones are the same as used in Figure 1. Similar results were obtained in repeated experiments. (**b**) Adenoviral transduction of MnSOD suppressed HIF-1α protein accumulation at 4% O_2. The top panel shows that MnSOD activity increased with increasing MOI (from 1.25 up to 200). Adenoviral transduction was performed at 21% O_2 and the transduced cells were exposed to 4% O_2. The second and third panels show Western blots for HIF-1α and α-tubulin, respectively, from cells exposed to 4% O_2 for 4 h. Similar results were obtained in repeat experiments

VEGF, mainly through the induction of the transcription factor HIF-1 (Kim *et al*., 2003). With the observation that MnSOD suppressed hypoxic accumulation of HIF-1α protein, the effect of increasing MnSOD activity on hypoxic induction of VEGF in MCF-7 cells was also measured.

Wild-type (WT) MCF-7 cells after hypoxic exposure (1% O_2) for various times were lysed and total RNA isolated to be used in both semiquantitative and real-time RT–PCR measurements. VEGF-A mRNA expression increased dramatically after cells were exposed to hypoxia for 6 h, compared to cells without hypoxia exposure. This induction continued in cells that were exposed for longer times (Figure 4a). To confirm these results, quantitative real-time RT–PCR for VEGF-A expression was also carried out using the primers described in Materials and methods (Figure 4b). The results also suggested that VEGF-A expression increased with time of exposure of cells to 1% O_2. Therefore, real-time RT–PCR confirmed that VEGF-A expression increased in MCF-7 WT cells after exposure to 1% O_2.

Hypoxic induction of VEGF-A mRNA was suppressed by increased MnSOD in cells exposed to 1% O_2 in stable transfectants

As VEGF-A mRNA expression increased substantially with 6 h of hypoxia, the effect of increased MnSOD

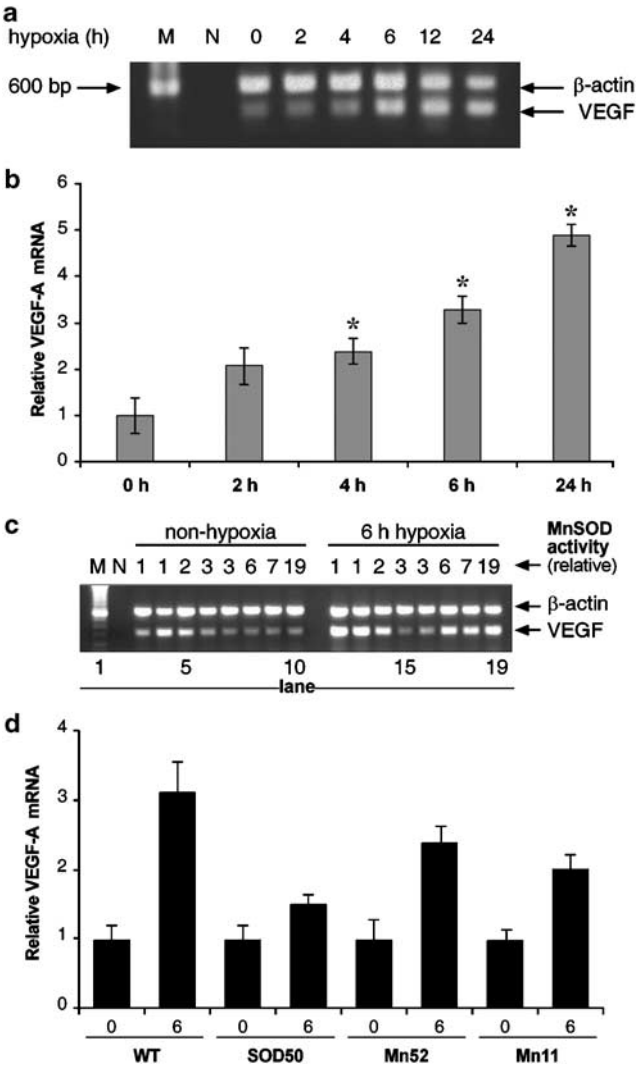

Figure 4 MnSOD overexpression suppressed hypoxic induction of VEGF-A mRNA in MCF-7 cells. (**a**) Steady-state levels of VEGF mRNA increased with time when MCF-7 WT cells were subjected to hypoxia. Cells were exposed to hypoxia (1% O_2) for various times. Total RNA was then isolated and semiquantitative RT–PCR was carried out. β-Actin was used as the internal control. Similar results were observed in repeated experiments. M: DNA marker; N: negative control (without mRNA samples); 0, 2, 4, 6, 12, and 24 h are different times of hypoxia exposure (1% O_2). (**b**) Quantitative RT–PCR analysis also documented an increase in VEGF-A expression with time (0–24 h) of exposure of MCF-7 WT cells to 1% hypoxia. The VEGF-A expressions in samples were normalized with 18S rRNA. Values are mean $\pm$ s.e. of three samples each from two separate experiments. $^*P<0.05$, group statistically different from 0 time controls by one-way ANOVA analysis. (**c**) A representative result shows that MnSOD overexpression suppressed hypoxic (1% O_2, 6 h) induction of VEGF mRNA in seven distinct MnSOD stably transfected clones as well as parental MCF-7 cells. M: DNA marker; N: negative control (without mRNA samples). (**d**) Quantitative RT–PCR analysis of VEGF-A expression in different MnSOD-overexpressing clones subjected to 6 h of hypoxia compared to cells grown at 21% (0 h of hypoxia). The bar graph illustrates that there is differential induction of VEGF-A expression in MCF-7 clones. Results are from measurements repeated three times from the same sample

activity on VEGF-A mRNA expression at this time was studied. Because MnSOD modulated HIF-1α and HIF-1α accumulation leads to VEGF expression, it was anticipated that MnSOD would modulate VEGF. Indeed, MnSOD transfection modulated VEGF-A expression with hypoxic exposure parallel to the observation with HIF-1α. After cells were exposed to 6 h hypoxia, VEGF-A mRNA expression was induced in parental cells. In cells with intermediate levels of MnSOD, the induction was suppressed (Figure 4c). To validate these findings, quantitative real-time RT–PCR was carried out with some of the clones that were subjected to hypoxia (1% O_2) and compared to that at 21% O_2 (Figure 4d). The VEGF-A expression in WT cells was more than three-fold increased after 6 h of hypoxia compared to cells under 21% O_2. VEGF expression was about 1.5-fold in clone SOD50 that has a three-fold increase in MnSOD activity. The clone that had a six-fold in MnSOD activity (Mn52) showed about a 2.4-fold increase in VEGF-A mRNA levels. In the clone with higher MnSOD activity (19-fold, Mn11) the VEGF-A expression was suppressed in hypoxia, but not to the level observed in SOD50 clones. These data show that VEGF-A mRNA levels are induced by hypoxia, but the levels are suppressed in MnSOD-overexpressing cells with again a biphasic effect.

MnSOD suppressed the secretion of VEGF protein with hypoxia stimulation (1% O_2)

As a mitogenic cytokine, VEGF protein is usually secreted outside the cells to stimulate the proliferation of endothelial cells (Dvorak *et al*., 1995b). To determine if VEGF protein secretion followed the trend of its mRNA expression, the pattern of VEGF protein secretion with hypoxia stimulation was examined. MCF-7 WT cells were exposed to hypoxia for various times and VEGF concentrations were measured. Hypoxia exposure increased the amount of VEGF protein (Figure 5a). The amount of VEGF protein increased rapidly from 8 until 12 h in the hypoxia-treated cells; after 12 h, VEGF levels continued to increase, but at a lower rate than earlier times. VEGF protein also increased in nonhypoxic cells, but at a much slower rate than in hypoxic cells. Moreover, most of the VEGF protein accumulated in the medium and only small amounts were found inside the cells (Figure 5a).

To determine the suppressive effect of elevated MnSOD activity on the secretion of VEGF protein, seven stably transfected MCF-7 clones with various MnSOD activity (described above) together with parental cells were exposed to hypoxia (1% O_2) for 12 h. Media from these clones were then collected and VEGF concentrations were measured. Increased MnSOD activity significantly decreased VEGF protein levels (Figure 5b). The suppression of VEGF protein by MnSOD was biphasic, with the greatest suppression at low to medium MnSOD levels and less suppression at higher MnSOD levels, consistent with what was observed with HIF-1α protein and VEGF mRNA levels.

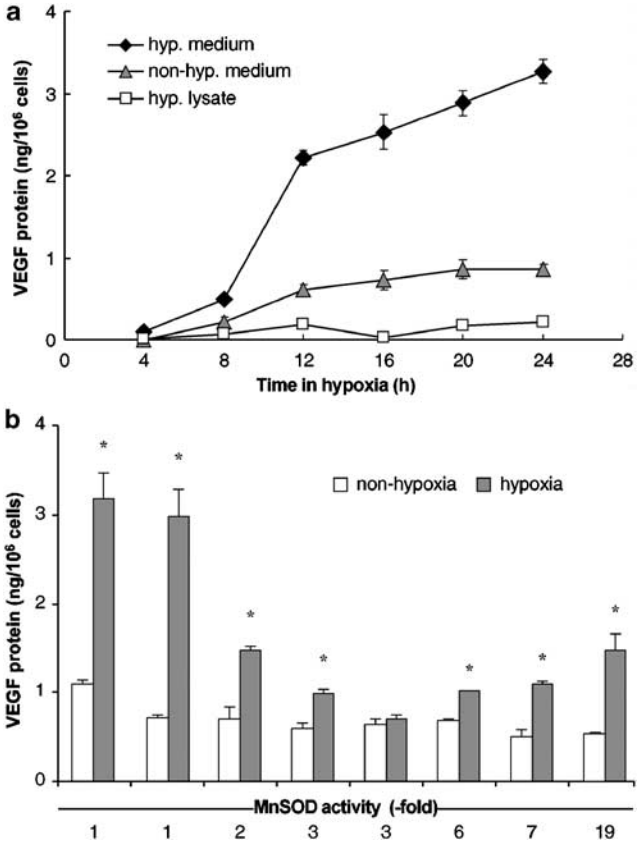

Figure 5 MnSOD overexpression suppressed VEGF protein secretion in MCF-7 cells. (**a**) Hypoxia induced the secretion of VEGF protein in a time-dependent manner in MCF-7 WT cells. MCF-7 WT cells were exposed to hypoxia (1% O_2) for various times. VEGF concentration was measured with a human VEGF-ELISA kit (R&D Systems, Minneapolis, MN, USA). VEGF production increased when cells were exposed to hypoxia for longer times. VEGF was mainly secreted outside the cell as the concentration of VEGF in tissue culture medium was much higher than that in the cell lysate. Values represent mean ± s.d. of three measurements of a single sample. (**b**) MnSOD overexpression suppressed VEGF protein secretion in MCF-7 cells. VEGF protein was measured after 12 h of hypoxia (1% O_2) in seven distinct clones plus MCF-7 parental cells with various MnSOD activities. Values represent mean ± s.e.m. of three independent experiments. *By Student's *t*-test analysis, hypoxia-treated cells were different from nonhypoxic cells at $P<0.05$ level

Adenoviral transduction of cellular catalase increased catalase activity in MCF-7 Neo vector control cells

Throughout this work, a biphasic effect was observed. One possible explanation for the stabilization of HIF-1α seen at high levels of MnSOD is that H_2O_2 produced from MnSOD overwhelms the endogenous peroxide-removing systems and causes HIF-1α to increase. In order to examine this hypothesis, extracellular H_2O_2 levels in these cell lines stably overexpressing MnSOD were measured. Cells overexpressing MnSOD did have higher levels of exogenous H_2O_2, but there was no difference between hypoxic and nonhypoxic cells (data not shown). This result suggested that H_2O_2 could be the effector of the biphasic effect since it was elevated in cells with high levels of MnSOD. Moreover, we have

shown in a previous paper that in the MCF-7 clones, the levels of intracellular H_2O_2 as measured by DCF-DA fluorescence increased as the levels of MnSOD increased (Zhang *et al*., 2002). If the effects seen at high levels of MnSOD are due to the measured increased levels of H_2O_2, then peroxide removal would be expected to eliminate the biphasic effect and prevent HIF-1α stabilization. Catalase (CAT) is one of the most intensively studied H_2O_2-removing enzymes. Therefore, it was decided to examine the effect of increasing CAT activities with the adenoviral transduction technique in MnSOD-overexpressing cells.

We first determined whether adenoviral transduction of human cellular catalase (cCAT) could increase CAT activity in MCF-7 cells, and at the same time, determined a proper dose for further experiments. Adenoviral cCAT cDNA was introduced into MCF-7 Neo vector control cells at increasing MOI (calculated from PFU). CAT activities were determined by running CAT activity gels. CAT activities increased with increasing MOI of adenoviral cCAT (Figure 6a). A dose of 100 MOI of adenoviral cCAT was used for further experiments.

Adenoviral transduction or inhibition of native cCAT did not alter the MnSOD suppressive effect on HIF-1α protein accumulation

Human cCAT (100 MOI) was introduced into MnSOD stably transfected MCF-7 clones. Besides MCF-7 WT and Neo, three other clones were used in these experiments: SOD50 with a three-fold increase in MnSOD activity compared to MCF-7 WT cells, Mn52 with a six-fold increase in MnSOD activity, and Mn11 with a 19-fold increase in MnSOD activity. We first showed that neither CAT protein nor activity was significantly different in the various lines (data not shown); moreover, CAT protein levels were not changed by hypoxia (data not shown). Cells were incubated with adenovirus carrying human CAT for 24 h. Adenovirus was removed and fresh medium was added onto cells for another 24 h. Cells were then exposed to hypoxia for 4 h to induce HIF-1α. Western blotting was used to detect HIF-1α protein levels in these clones.

Without CAT transduction, these five clones showed the biphasic suppressive effect of increased MnSOD expression on HIF-1 induction (Figure 6b). That is, HIF-1α protein accumulated in MCF-7 WT and Neo clones with low MnSOD activity. In clones with medium MnSOD activity (SOD50 and Mn52), the accumulation of HIF-1α protein was suppressed, whereas in the clone with high MnSOD activity (Mn11), HIF-1α protein again accumulated. Contrary to what was expected, cCAT transduction did not cause significant changes in HIF-1α protein levels in any of these clones (Figure 6b and c). Increasing CAT activity by transducing CAT did not alter the suppressive effect of MnSOD.

Since MCF-7 cells have relatively high endogenous CAT activity, adding more CAT into the cells may not show any significant effect. Therefore, the effect of inhibiting endogenous CAT was next examined.

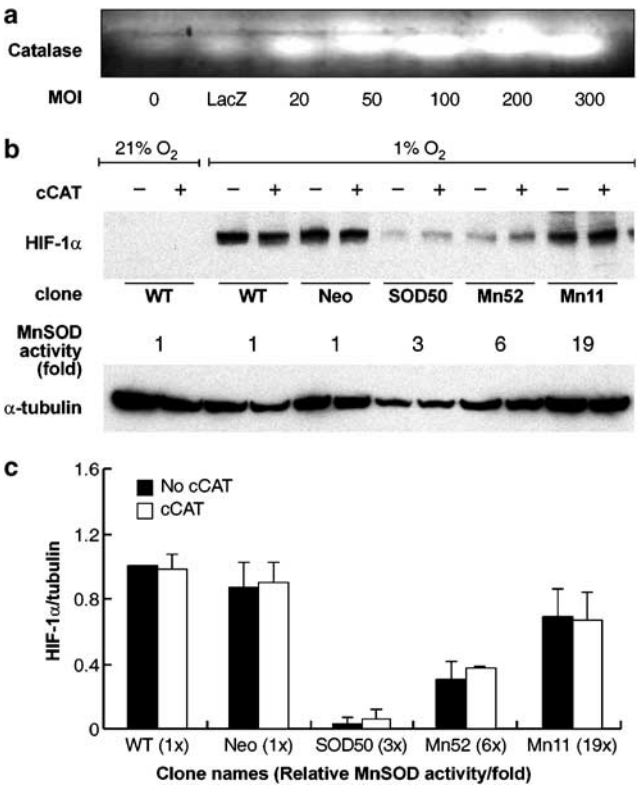

Figure 6 (**a**) Adenoviral transduction of peroxisomal CAT/cCAT increased CAT activities in MCF-7 Neo vector control cells. Cells (1×10^6) were seeded in 100 mm^2 tissue culture dishes. Adenoviral cCAT was added onto the cells at various MOI, which was calculated from PFU. After 24 h of infection, medium containing adenovirus was removed and fresh medium was added onto the cells. Cells were then allowed to recover for 24 h. After recovery, cells were scraped and centrifuged to obtain a cell pellet. Cell lysates were then obtained by sonication. CAT activity gel assay was carried out. A 30 μg portion of protein per lane was used in this experiment. The experiment was repeated with similar results. (**b**) Adenoviral transduction of human CAT (cCAT) did not alter the MnSOD suppressive effect on HIF-1α protein accumulation. Human CAT (cCAT, 100 MOI) was introduced into control cells and four MnSOD stably transfected MCF-7 clones: MCF-7 WT, Neo, SOD50 (three-fold increase in MnSOD activity compared to MCF-7 WT), Mn52 (six-fold increase in MnSOD activity), and Mn11 (19-fold increase in MnSOD activity). Cells were incubated with adenovirus carrying cCAT for 24 h. Medium containing adenovirus was removed and fresh medium was added onto cells for another 24 h. Cells were then exposed to hypoxia for 4 h to induce HIF-1α. Western blot was used to detect HIF-1α protein levels in these clones. α-Tubulin was used as an internal control. (**c**) Densitometric analysis of the Western blot. Values represent mean ± s.d. $n = 3$ different samples. No group was statistically different with transduction *vs* without transduction

3-Amino-1,2,4-triazole (AT) is an irreversible inhibitor of CAT activity (Darr and Fridovich, 1986). In order to determine the role of endogenous CAT in mediating the suppressive effect of MnSOD, MCF-7 cells were treated with AT (18 mM, 24 h) to decrease endogenous CAT activity. Under both 21 and 1% O_2 conditions, CAT activities decreased to less than 20% of the original activities in all cells treated with AT (data not shown). Cells were then exposed to hypoxia (1% O_2) for 4 h. Western blotting was carried out to determine HIF-1α

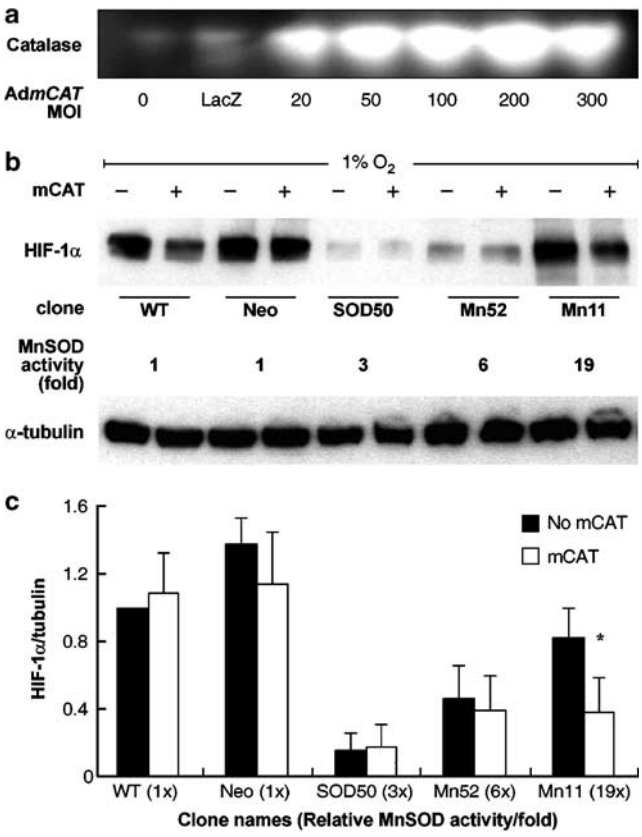

Figure 7 (**a**) Adenoviral transduction of mCAT increased CAT activities in MCF-7 Neo vector control cells. Cells (1×10^6) were seeded in 100 mm^2 tissue culture dishes. Adenoviral mCAT was added onto the cells at various MOI, which was calculated from PFU. After 24 h of infection, medium containing adenovirus was removed and fresh medium was added onto the cells. Cells were then allowed to recover for 24 h. After 24 h of recovery, cells were scraped and centrifuged to obtain a cell pellet. Cell lysates were then obtained by sonication. CAT activity gel assay was carried out. A 30 μg portion of protein per lane was used in this experiment. The experiment was repeated with similar results. (**b**) Adenoviral transduction of mCAT decreased HIF-1α protein levels in clones with high MnSOD activity. mCAT (50 MOI) was introduced into control cells and four of the MnSOD stably transfected MCF-7 clones: MCF-7 WT, Neo, SOD50 (three-fold increase in MnSOD activity compared to MCF-7 WT), Mn52 (six-fold increase in MnSOD activity), and Mn11 (19-fold increase in MnSOD activity). Cells were incubated with adenovirus carrying mCAT for 24 h. Medium containing adenovirus was removed and fresh medium was added onto cells for another 24 h. Cells were then exposed to hypoxia for 4 h to induce HIF-1. Western blot was used to detect HIF-1α protein levels in these clones. α-Tubulin was used as an internal control. HeLa cell lysate was used as HIF-1α protein positive control. (**c**) Densitometric analysis of the Western blot. Values represent mean $\pm$ s.d. of results from three independent samples. *mCAT-treated group was statistically different from the untreated group at the $P<0.05$ level

protein changes. Similar to increasing CAT activity with adenoviral transduction, CAT inhibition induced by AT did not cause significant changes in HIF-1α protein levels in MnSOD-overexpressing clones (data not shown). These results suggested that peroxide removal by CAT did not play a role in mediating the suppressive effect of MnSOD on hypoxic induction of HIF-1. Since MnSOD is located in the mitochondria and CAT removes peroxide outside the mitochondria in either peroxisomes or cytoplasm, the location of the peroxide-removing system may be important and responsible for these negative results.

Adenoviral transduction of mitochondrial catalase increased CAT activity in MCF-7 Neo vector control cells

Since neither native CAT transduction nor CAT inhibition changed the suppressive effect of MnSOD, a human adenoviral CAT construct with a mitochondrial targeting sequence was obtained to test whether the location of the peroxide-removing system was important.

Adenoviral mitochondrial catalase (mCAT) was made by Dr Shawn Flanagan from a plasmid kindly given to us by Dr Andres Melendez of Albany Medical College. The recombinant mCAT cDNA contains an added mitochondrial targeting sequence, but the peroxisomal targeting sequence has not been removed. Thus, the protein is expected to be found in both mitochondria and peroxisomes; this has been confirmed in published studies (Bai *et al*., 1999; Rodriguez *et al*., 2000). It was first determined whether adenoviral transduction of mCAT could increase CAT activity in MCF-7 cells, and at the same time, a proper dose for further experiments was ascertained. Adenoviral mCAT cDNA was introduced into MCF-7 Neo vector control cells at increasing MOI (calculated from PFU). CAT activity increased with adenovirus-mediated gene transfer (Figure 7a). High levels of CAT activity were achieved with as low as 20 MOI. A dose of 50 MOI of adenoviral mCAT was used for further experiments.

Adenoviral transduction of mCAT decreased HIF-1α protein levels in a clone with high MnSOD activity

Adenoviral mCAT (50 MOI) was introduced into MnSOD stably transfected MCF-7 clones. In addition to MCF-7 WT and Neo, three other clones were used in these experiments: SOD50 with a three-fold increase in MnSOD activity compared to MCF-7 WT cells, Mn52 with a six-fold increase in MnSOD activity, and Mn11 with a 19-fold increase in MnSOD activity. Cells were incubated with adenovirus carrying mCAT for 24 h. Adenovirus was removed and fresh medium was added to cells for another 24 h. Cells were then exposed to hypoxia for 4 h to induce HIF-1α. Western blotting was used to detect HIF-1α protein levels in these clones.

In MCF-7 WT and Neo cells, the transduction of mCAT did not alter HIF-1α protein levels. In clones with medium MnSOD activity, clone SOD50 (three-fold) and clone Mn52 (six-fold), HIF-1α protein levels did not change with mCAT transduction (Figure 7b). Interestingly, HIF-1α protein levels decreased in clone Mn11 with mCAT transduction compared to the same cells without mCAT transduction (Figure 7b and c). Mn11 is the clone with high MnSOD activity (19-fold). Densitometric analysis showed that HIF-1α protein

levels decreased significantly in Mn11 after the transduction of mCAT to a level of about 50% of the untransduced cells (Figure 7c).

Adenoviral transduction of glutathione peroxidase-1 increased GPx activity in MCF-7 Neo vector control cells

The transduction of native cCAT did not cause any change in HIF-1α protein levels in any of the clones, whereas the transduction of mCAT decreased the HIF-1α protein levels in the clone with high MnSOD activity. These results indicate that the location of the peroxide-removing system is important to be able to alter the suppressive effect of overexpressed MnSOD. Therefore, the effect of transducing another peroxide-removing enzyme, glutathione peroxidase-1 (GPx-1), was tested. An immunogold assay had previously verified that the adenoviral GPx-1 entered both cytosol and mitochondria (Li *et al*., 2000). It was first determined whether adenoviral transduction of GPx-1 could increase GPx activity in MCF-7 cells, and at the same time, a proper dose for further experiments was ascertained. Adenoviral GPx-1 cDNA was introduced into MCF-7 Neo vector control cells at increasing MOI (calculated from PFU). GPx activity increased with adenovirus-mediated gene transfer (Figure 8a). Because high levels of GPx activity were achieved with 100 MOI, a dose of 100 MOI of adenoviral GPx was used for further experiments.

Adenoviral transduction of GPx-1 decreased HIF-1α protein levels in a clone with high MnSOD activity

Adenoviral GPx-1 (100 MOI) was introduced into MnSOD stably transfected MCF-7 clones. Besides MCF-7 WT and Neo, three other clones were used in these experiments: SOD50 with a three-fold increase in MnSOD activity compared to MCF-7 WT cells, Mn52 with a six-fold increase in MnSOD activity, and Mn11 with a 19-fold increase in MnSOD activity. All of these lines had nondetectable GPx activity as measured by activity gels before transduction in cells grown in either 21 or 1% oxygen (data not shown). Cells were incubated with adenovirus carrying GPx-1 for 24 h. Adenovirus was removed and fresh medium was added onto cells for another 24 h. Cells were then exposed to hypoxia for 4 h to induce HIF-1α. Western blotting was used to detect HIF-1α protein levels in these clones.

In MCF-7 WT and Neo cells, the transduction of GPx-1 did not alter HIF-1α protein levels. In clones with medium MnSOD activity, clone SOD50 (three-fold) and clone Mn52 (six-fold), HIF-1α protein levels did not change with GPx-1 transduction (Figure 8b). Similar to what was observed with mCAT transduction, HIF-1α protein levels decreased in clone Mn11 after the adenoviral infection of GPx-1 (Figure 8b). Mn11 is the clone with high MnSOD activity (19-fold). Densitometric analysis showed that HIF-1α protein levels decreased significantly in Mn11 after the transduction of GPx-1 to a level about 75% less than untransduced cells (Figure 8c).

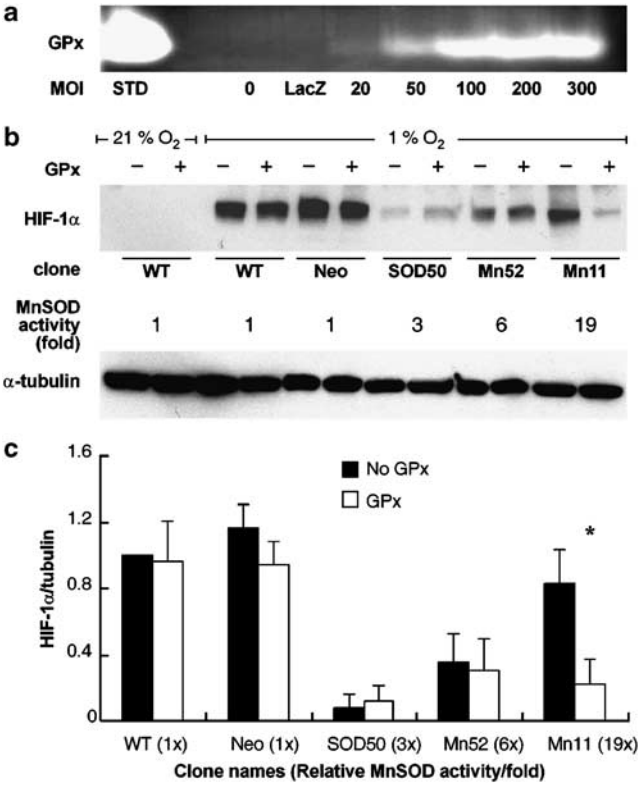

Figure 8 (**a**) Adenoviral transduction of GPx-1 increased GPx activity in MCF-7 Neo vector control cells. Cells (1×10^6) were seeded in 100 mm^2 tissue culture dishes. After cells were attached, adenoviral GPx-1 (100 MOI) was added onto the cells at various MOI, which was calculated from PFU. After 24 h of infection, medium containing adenovirus was removed and fresh medium was added onto the cells. Cells were then allowed to recover for 24 h. Then, cells were scraped and centrifuged to obtain cell pellets. Cell lysates were then obtained by sonication. GPx activity gel assay was then carried out (150 μg protein per lane was used in this experiment). STD is pure human GPx protein. The experiment was repeated with similar results. (**b**) Adenoviral transduction of GPx-1 decreased HIF-1α protein levels in clones with high MnSOD activity. Adenoviral GPx-1 (100 MOI) was introduced into control cells and four of MnSOD stably transfected MCF-7 clones: MCF-7 WT, Neo, SOD50 (three-fold increase in MnSOD activity compared to MCF-7 WT), Mn52 (six-fold increase in MnSOD activity), and Mn11 (19-fold increase in MnSOD activity). Cells were incubated with adenovirus carrying GPx-1 for 24 h. Medium containing adenovirus was removed and fresh medium was added onto cells for another 24 h. Cells were then exposed to hypoxia for 4 h to induce HIF-1α. Western blot was used to detect HIF-1α protein levels in these clones. α-Tubulin was used as an internal control. (**c**) Densitometric analysis of the Western blot. Values represent mean ± s.d. of results from three independent samples. *GPx-treated group was statistically different from the untreated group at the $P < 0.05$ level

Discussion

Hypoxia is a deficiency in oxygen (Ozaki *et al*., 1999; Semenza *et al*., 1999). Hypoxia is widespread in solid tumors due to an inefficient vascular supply of oxygen. HIF-1 is an important transcription factor that is activated in conditions of decreased oxygen (Semenza *et al*., 1998). HIF-1 mediates cell survival in hypoxia by promoting genes involved in glucose homeostasis, erythropoiesis, and angiogenesis (Semenza,

1999). While normal tissue and benign breast tumors do not exhibit increased HIF-1 activity, evidence for a graded increase in activity has been demonstrated in the progression from preneoplastic lesions to cancer metastases (Bos *et al*., 2001). Moreover, a positive correlation has been found between HIF-1 expression and vascularization in brain tumors (Zagzag *et al*., 2000).

As one of the HIF-1 downstream genes, VEGF is a pivotal mitogen that mediates endothelial cell proliferation and new vessel formation, a process known as angiogenesis. Hypoxic induction of VEGF is mediated by HIF-1 (Forsythe *et al*., 1996).

MnSOD is an important antioxidant enzyme involved in cancer cell growth. A change in the level of MnSOD enzyme activity will change the redox status of the cell and affect the expression of redox-sensitive genes and proteins. HIF-1 is known to be a redox-sensitive protein, but the effect of MnSOD on HIF-1 expression has not been studied until the present work (Huang *et al*., 1996; Chandel *et al*., 2000; Kelley and Parsons, 2001). It has been shown both *in vitro* and *in vivo* that tumor growth is suppressed with MnSOD overexpression (Li *et al*., 1998a, b; Zhang *et al*., 1999). MnSOD has been proposed as a tumor suppressor gene (Oberley and Oberley, 1988; Bravard *et al*., 1992). MnSOD catalyses the reaction $2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$. It is well accepted that a change in MnSOD activity will result in a change in the steady-state level of superoxide ($O_2^{\bullet-}$). The fact that a moderate increase in MnSOD decreased the accumulation of HIF-1α suggests an involvement of superoxide. It has been observed that a moderate increase in nitric oxide ($NO^{\bullet}$) decreased HIF-1α accumulation (Mateo *et al*., 2003; Thomas *et al*., 2004). Because $NO^{\bullet}$ reacts rapidly with $O_2^{\bullet-}$, an increase in $NO^{\bullet}$ will decrease the steady-state level of $O_2^{\bullet-}$, similar to an increase in MnSOD activity. These observations suggest that superoxide could modulate HIF-1α accumulation.

However, a product of MnSOD enzymatic activity, H_2O_2, is a highly diffusible molecule that moves freely across cell membranes. H_2O_2 also contributes to the redox environment of the cell (Oberley and Buettner, 1979). Therefore, H_2O_2 may also be a link between MnSOD and HIF-1α. Both superoxide and H_2O_2 could react with the iron of HIF-prolyl hydroxylase, modulating its activity and affecting the accumulation of HIF-1α. However, superoxide in general causes reduction of iron, while H_2O_2 will oxidize it:

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$$

If Fe^{2+} is necessary for HIF-1α-prolyl hydroxylase (PH) activity, then superoxide radical should increase PH activity, and H_2O_2 should decrease PH activity. Thus, superoxide radical should decrease the levels of HIF-1α, while H_2O_2 should increase the levels of HIF-1α. Our data as discussed below show just this for H_2O_2, but the opposite for superoxide radical: the higher the SOD levels and thus the lower the levels of superoxide radical, the lower the levels of HIF-1α, until very high levels of SOD are reached.

We have shown here that MnSOD suppressed hypoxic accumulation of HIF-1α protein, which led to lower levels of VEGF. Moderate levels of MnSOD activity (two- to six-fold increase compared to parent cells) in MCF-7 cells abolished the hypoxic accumulation of HIF-1α protein and the induction of VEGF. Surprisingly, higher levels of MnSOD allowed HIF-1α protein accumulation, showing a biphasic modulation on HIF-1α accumulation. Interestingly, in these high MnSOD-overexpressing cells, HIF-1α never returned to values near those of the parent cells; in other words, HIF-1α was still suppressed relative to parent cells and vector control. We hypothesize that elevation of MnSOD to moderate levels (physiological levels for most normal tissues; see Oberley *et al*., 1989) will lower the steady-state level of superoxide, which then lowers HIF-1α levels. However, if MnSOD is increased above these levels, H_2O_2 could be a modulator. An amount of peroxide may be produced that is above the levels that the endogenous peroxide-removing capabilities of the cell can accommodate. Too much H_2O_2 then leads to inhibition of HIF-1α degradation and HIF-1α accumulates. As mentioned above, this effect of high MnSOD could be due to oxidation of iron, removing the required Fe^{2+} and also producing the damaging hydroxyl radical.

It is well accepted that increasing SOD levels lowers the steady-state levels of superoxide radicals, but there is still controversy over whether the levels of H_2O_2 increase (Omar and McCord, 1990; Li *et al*., 2000). We have argued that in cells, H_2O_2 levels must increase after increases in MnSOD based on three observations: (1) after stable overexpression of MnSOD, in many clones either CAT or GPx levels are found to increase also (Liu *et al*., 1997; Zhong *et al*., 1997; Li *et al*., 1998b); (2) stable expression of either CAT (Rodriguez *et al*., 2000) or GPx (Li *et al*., 2000) modulates the effects of MnSOD overexpression; and (3) assays of either intracellular (Zhang *et al*., 2002) or extracellular H_2O_2 (Wenk *et al*., 1999) concentrations show increases after MnSOD overexpression. The problem with the measurements of H_2O_2 is that the extracellular measurements are quite specific, but it is unclear where the H_2O_2 is coming from, and the intracellular measurements are made with nonspecific assays. We have measured both intracellular and extracellular H_2O_2 in the cells used in the present work. Using dihydrofluorescein diacetate, we found that fluorescence increased in the stable MnSOD-overexpressing MCF-7 cell lines as the MnSOD activity increased (Zhang *et al*., 2002). The fluorescence was most likely due to intracellular H_2O_2 since it was blocked by adenoviral CAT or GPx and by the GPx mimic ebselen (Zhang *et al*., 2002). Moreover, in the present study, we measured extracellular H_2O_2 in these cell lines and found that the levels increased with increasing MnSOD activity (data not shown). Thus, the levels of both intracellular and

extracellular H_2O_2 appear to increase as the MnSOD levels increase. The reason why H_2O_2 levels increase after MnSOD overexpression is still the subject of intense study.

Another possibility for the effecter of MnSOD overexpression is molecular oxygen. MnSOD produces both H_2O_2 and O_2. Moreover, reduction of Fe^{3+} by superoxide also produces O_2 as shown above. HIF-1α degradation ensues upon the hydroxylation of prolines as accomplished by HIF-prolyl hydroxylase. This specific prolyl hydroxylase requires 2-oxoglutarate, Fe^{2+}, ascorbate, and molecular oxygen for enzymatic activity (Jaakkola *et al*., 2001; Kondo and Kaelin, 2001; Semenza, 2001a; Pugh and Ratcliffe, 2003b). All of these cofactors can be found in mitochondria as well as cytoplasm. Thus, the oxygen produced by MnSOD is also a possible effecter for the MnSOD suppressive effect we have observed. A mitochondrial localization for the effects we have observed is suggested because (1) MnSOD is known to be found in the mitochondria and (2) the induction of HIF-1 accumulation was blocked by CAT when it was found in mitochondria, but not by native peroxisomal CAT.

Our results are consistent with what we have observed with tumor incidence and tumor growth in nude mice after MnSOD overexpression (Zhang *et al*., 1999). That is, the incidence of tumors and growth of the tumors that do form decrease when MnSOD activity is increased to a moderate level ($<$6-fold). However, MnSOD does not further suppress the tumor formation when its activity is increased to a higher level ($>$6-fold), and indeed some of the tumor suppression effect is lost (Zhang *et al*., 1999). This suggests that the *in vivo* tumor suppressive function of MnSOD may be achieved by the inhibition of HIF-1 protein accumulation. By controlling HIF-1 protein and thereby controlling the expression of HIF-1 downstream genes that are involved in tumor metabolism and growth, such as VEGF, MnSOD may control a switch for genes that allow tumor growth and progression. These results suggest that MnSOD may inhibit tumor angiogenesis and that part of the tumor suppressive effect seen *in vivo* may be due to inhibition of angiogenesis.

In support of this proposal, Wheeler *et al*. (2003) have shown that adenovirus transduction of SOD3 (extracellular SOD or ecSOD) inhibits the growth of B16 melanoma cells in mice. At 2 weeks after implantation, B16 tumor size was 65% smaller in mice infected with Ad*ecSOD* in comparison with mice infected with Ad*lacZ*. Tumors from Ad*ecSOD*-infected mice expressed less VEGF protein. Importantly, blood vessel density as assessed by two different ways was markedly reduced in tumors from Ad*ecSOD*-infected mice compared with controls. Wheeler *et al*. did not examine the effects of MnSOD overexpression and did not determine if HIF-1 was involved. However, this study is consistent with our findings of the dramatic effect of MnSOD overexpression on HIF-1α accumulation and VEGF expression.

Last, a strong inducer of MnSOD is TNF-α (Wong *et al*., 1989). Therefore, one would expect that TNF-α would induce MnSOD in certain cells, leading to lowered levels of HIF-1α and VEGF. Future work is needed to investigate the role of cytokines in the regulation of this pathway.

Materials and methods

Cell culture

Human breast adenocarcinoma MCF-7 cells were routinely cultured in Eagle's MEM containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10% FBS, and incubated at 37°C with 95% air and 5% CO_2. MCF-7 clones that were stably transfected with MnSOD and Neo were cultured in the same medium supplemented with 400 μg/ml G418 (Life Technologies Inc.). The medium was generally changed every 3–4 days.

Hypoxic treatment

Cells were seeded into 60 mm tissue culture dishes (Corning Scientific Products DIV, Acton, MA, USA) and the medium was replaced with fresh medium before hypoxic treatment. The dishes were transferred to a modular incubator chamber (Billups-Rothenberg, Del Mar, CA, USA) that was flushed with either 1% O_2 (1% O_2, 5% CO_2 and balanced with N_2) or 4% O_2 (4% O_2, 5% CO_2 and balanced with N_2) for 4 min at a rate of 20 l/min, then sealed and placed at 37°C. If longer incubation times were necessary, gas was replaced at 6 h intervals.

Adenovirus infection

Adenoviral MnSOD, adenoviral cCAT, and adenoviral GPx were made originally in the laboratory of Dr John Engelhardt (Zwacka *et al*., 1998; Li *et al*., 2001) and were manufactured at The University of Iowa's Vector Core Facility or Viraquest Inc. (North Liberty, IA, USA). The original AdGPx produced a product with a myc tag (Li *et al*., 2001), but in the present work the AdGPx was modified so that it produced a native GPx without a myc tag. The mCAT plasmid was obtained from Dr Andres Melendez and was made into an adenovirus construct by Viraquest Inc. with the help of Dr Shawn Flanagan. For Western and activity gel analyses, MCF-7 cells were plated into 60 mm dishes at a density of 5×10^5 cells/plate in full media. The next day, adenovirus (prepared in 3% sucrose/phosphate-buffered saline (PBS)) was added to the cells containing 2 ml of media in each dish. To the appropriate dishes, a dose of 1.25–200 MOI of adenoviral constructs was added. The MOI for all experiments was calculated from PFU, which was determined by the Vector Core or Viraquest Inc. Following a 24 h incubation, the viral particles were removed and fresh medium was added. All infections were carried out at 21% O_2. Exposure to 1 and 4% O_2 was carried out at 24 h after adenoviral removal.

Adenoviral transduction efficiency

MCF-7 cells (2×10^4) were plated into eight-well slides (Nunc) and allowed to attach overnight. Various titers of Ad*MnSOD* (1.25–200 MOI) or Ad*LacZ* (200 MOI) were added for 24 h. Media were changed at 24 h. Cells were fixed with 4% gluteraldehyde at 48 h. Cells were incubated with human MnSOD primary antibody and goat anti-rabbit Alexa 488 secondary antibody (Molecular Probes). The cell images were captured and analysed with Image J. A total of 500 cells were analysed for each MOI from three to four fields. The threshold

for positive fluorescent staining was set at half the maximal pixel intensity. All cells below the threshold were not scored. The number of positively stained cells divided by the total number of cells was used to determine the percent immunofluorescence per sample.

Cell homogenization for SOD activity gel and SOD Western blot

Cells were washed in PBS (KCl 2.7 mM, KH_2PO_4 1.5 mM, $NaHPO_4$ 8 mM, and NaCl 136.9 mM, pH 7.0), scrape harvested, and pelleted at 12 000 *g* for 10 s in 1.5 ml microfuge tubes. The supernatant was removed and cells were resuspended in 50 mM phosphate buffer (PB, pH 7.8) and sonicated on ice for 3×30 s using a Vibra Cell cup horn sonicator (Sonics and Materials Inc., Danbury, CT, USA) at maximum power. Protein concentration was estimated by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) and standardized with bovine serum albumin.

Antioxidant protein activity gels

In this technique, nondissociating electrophoresis gels are run (Tulchin *et al*., 1976) with ammonium persulfate used as the initiator in the running gel (12.5%) and riboflavin-light in the stacking gel (5%). Once run, the gels were stained for SOD activity (Beauchamp and Fridovich, 1971). The gels were stained for CAT or GPx activity according to a previously published method (Sun *et al*., 1988).

MnSOD activity assay

SOD activities were determined using the modified nitroblue tetrazolium (NBT) method described earlier (Oberley and Spitz, 1984; Spitz and Oberley, 1989). The competition reaction between SOD and the superoxide indicator molecule, NBT, is the basis of this indirect assay. Xanthine/xanthine oxidase was used to generate superoxide, the substrate of SOD. Different amounts of total protein sample were added to the reaction until the maximal inhibition of the reaction was obtained as measured by a spectrophotometer at 560 nm. Total SOD activity was determined by the amount of protein necessary for half-maximal inhibition of the NBT reaction. MnSOD activity was quantified in the presence of 5 mM sodium cyanide, which inhibits CuZnSOD activity. One unit of activity was defined as the concentration of SOD that reduced the NBT reaction to one-half of the maximum.

SOD Western blot

The amount of immunoreactive MnSOD protein was measured by Western blotting (Oberley *et al*., 1989). Briefly, cell homogenates (30 μg) were processed by SDS–PAGE and transferred to PVDF (Millipore Corporation, Bedford, MA, USA) membranes. The membrane was then probed with rabbit anti-MnSOD IgG (1 : 1000 dilution). The secondary antibody was goat anti-rabbit IgG (Pharmingen/Transduction Laboratories, San Diego, CA, USA) used at 1 : 5000 dilution. Blots were visualized using Chemiluminescent Developer (Pierce, Rockford, IL, USA) and exposed to film. The bands were examined with a computerized digital imaging system using AlphaImager 2000 software (Alpha Innotech, San Leandro, CA, USA). The integrated density values (IDV) were obtained by integrating all of the pixel values in the area of one band after correction for background.

Protein harvest for HIF-1α Western blot

The medium was removed from tissue culture dishes. After rinsing twice with PBS, 100 μl of boiling lysis buffer (1% SDS, 1.0 mM sodium orthovanadate, and 10 mM Tris pH 7.4) was added to the cells. Cell lysates were then scraped and transferred into microcentrifuge tubes and boiled for 5 min. Protein concentrations were determined with the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) as described by the manufacturer.

HIF-1α Western blot

A 30 μg portion of total protein was separated on a 4–20% gradient Tris-HCl polyacrylamide ready gel (Bio-Rad Laboratories, Hercules, CA, USA). Then, the protein was electrotransferred onto a PVDF membrane (Millipore Corporation, Bedford, MA, USA) by running at 100 V for 1 h. For HIF-1α Western blot, the primary antibody was mouse anti-HIF-1α IgG (Pharmingen/Transduction Laboratories, San Diego, CA, USA) used at 1 : 2000 dilution. For α-tubulin Western blot, the primary antibody was mouse anti-human α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) used at 1 : 5000 dilution. The secondary antibody used against both primary antibodies was goat anti-mouse IgG (Pharmingen/Transduction Laboratories, San Diego, CA, USA) used at 1 : 2000 dilution. Blots were visualized using Chemiluminescent Developer (Pierce, Rockford, IL, USA) and exposed to film. The bands were examined with a computerized digital imaging system using AlphaImager 2000 software (Alpha Innotech, San Leandro, CA, USA). The IDV were obtained by integrating all of the pixel values in the area of one band after correction for background.

Semiquantitative RT–PCR analysis

Total RNA was extracted by the Qiagen RNeasy kit (Qiagen Inc., Valencia, CA, USA) as recommended by the manufacturer. RT–PCR was carried out using a Qiagen One-Step RT–PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendation. The RT–PCR product was electrophoresed on a 1% agarose gel.

Analysis of VEGF-A expression using quantitative real-time RT–PCR

The isolated RNA was reverse transcribed using the cDNA High-Capacity Archive kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Quantitative real-time RT–PCR was performed on an ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA) using the TaqMan PCR master mix (Applied Biosystems, Foster City, CA, USA) . Primers for amplification of VEGF-A cDNA were designed using Primer Express (ABI) and were 5′-CTC TAC CTC CAC CAT GCC AAG-3′ (forward) and 5′-AGA CAT CCA TGA ACT TCA CCA CTT C-3′ (reverse) and were obtained from IDT Inc. (Coralville, IA, USA). The PCR reactions were performed in 50 μl volumes containing 150 ng cDNA, 270 nM of forward and reverse primers for VEGF-A, 0.09 μM fluorescent VEGF-A probe (IDT Inc.), and 2x-TaqMan PCR master mix. The PCR sequential thermal cycling profile was as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 s with a final extension of 60°C for 1 h. Since amplification products were detected using TaqMan, dissociation curves were performed to verify specificity of the products formed during PCR (data not shown). 18S rRNA was measured using the SYBR Green PCR master mix (ABI) and the expression level of VEGF-A was normalized to 18S rRNA.

H_2O_2 measurement

The concentrations of H_2O_2 were determined with the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, OR, USA). MCF-7 WT and other clones were exposed to hypoxia (1% O_2) for 0.5, 1, and 2 h. Right after hypoxic exposure, PB on the cells was collected and the concentration of H_2O_2 was determined by measuring resorufin with absorbance at $\sim$560 nm. Cells that were exposed to nonhypoxia (21% O_2) for the corresponding times were used as controls.

Statistics

Pearson's regression and correlation analysis were used to determine the relationship between the MnSOD activities and HIF-1α protein levels. The R^2 and *P*-value were calculated using Microsoft Excel software. Student's *t*-test for two samples assuming equal variance was used for the comparison of HIF-1α and VEGF protein levels between MCF-7 WT cells and MnSOD-transfected clones, and for the comparison of HIF-1α protein levels after the transduction of GPx-1, cCAT, mCAT, or AT treatment. To estimate statistical difference in the induction of VEGF in WT cells with time, one-way ANOVA with Bonferroni correction was performed using the SAS software program.

Abbreviations

HIF-1, hypoxia-inducible factor-1; MnSOD, manganese superoxide dismutase; MOI, multiplicity of infection; NBT, nitroblue tetrazolium; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau.

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